Comparative Molecular Characterisation of Inherited Disease Models of Cancer and Cardiovascular Disease

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Abstract

Heart disease and cancer are leading causes of global non-communicable morbidity and mortality. There have been improvements in diagnosis, treatment, and survival for many heart diseases and cancers. There is, however, considerable potential for improvements in the prevention, diagnosis, and management of dilated cardiomyopathy (DCM) and osteosarcoma. There is currently no cure for DCM, and osteosarcoma survival rates have not improved in decades. New diagnostic tools and treatments are urgently needed for these diseases.

In this thesis, the overarching hypothesis is that diseases in the companion animal population represent good models of human disease, whilst also representing important veterinary clinical challenges. Specific hypotheses relating to the genetics of canine DCM and osteosarcoma were tested. Both diseases have high prevalence rates in certain breeds, in particular Irish Wolfhounds develop both DCM and osteosarcoma. As the same breeds are affected by both diseases it was proposed that a common mechanism might influence the development of both diseases in these dogs. To test whether this association was due to differences in androgen signalling the polyglutamine repeat tracts of the androgen receptor and *NCOA3* genes were genotyped. Neither disease was associated with polyglutamine repeat tract length, but this does not exclude endocrine signalling as a common factor influencing canine DCM and osteosarcoma development. Genetic associations specific to each disease were also examined with a multigenic model of DCM developed in Doberman Pinschers and further tested in Irish Wolfhounds. This showed that multiple loci act together to influence DCM development and better predict disease than individual loci.

In addition to testing hypotheses relating to the genetics of DCM it was hypothesised that the fatty acid profiles of canine DCM patients would be different from other cardiac patients and non-cardiac patients. If differences can be identified it could lead to improved diagnosis and treatment, but also give indications as to which genes could be involved in disease pathology. Results

presented in this thesis show that there is indeed a difference in fatty acid profiles between cardiac and non-cardiac patients, but that it is valve disease patients that appear different from all other patients. The transcriptome of osteosarcoma and non-malignant tissue was compared to identify novel biomarkers of disease with several interesting genes identified as differentially expressed in tumours compared to non-tumour tissue. Additional work is required to establish the nature of the disease process that is shared between canine DCM and osteosarcoma, and to identify additional genetic loci associated with disease.

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List of Abbreviations

6- FAM	6-Carboxyfluorescein
9-HODE	9-Hydroxyoctadecadienoic Acid
ACE	Angiotensin Converting Enzyme
AF	Atrial Fibrillation
ANOVA	Analysis of Variance
AR	Androgen Receptor
ARVC	Arrhythmic Right Ventricular Cardiomyopathy
BLAST	Basic Local Alignment Search Tool
BP	Base Pairs
CAD	Coronary Artery Disease
cDNA	complementary DNA
COX	Cyclooxygenase
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTGF	Connective Tissue Growth Factor
dbSNP	The Single Nucleotide Polymorphism Database
DCM	Dilated Cardiomyopathy
DNA	Deoxyribonucleic Acid
ERα	Estrogen Receptor Alpha
ERβ	Estrogen Receptor Beta
FOXO4	Forkhead Box O4
GO	Gene Ontology
GTR	Genetic Testing Registry
GWAS	Genome Wide Association Study
HEX	Hexachloro-fluorescein
IMS	Industrial Methylated Spirits
IWDB	Irish Wolfhound Database
IWH	Irish Wolfhound
IRF8	Interferon Regulatory Factor 8
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDL	Low Density Lipoprotein
LEF1	Lymphoid Enhancer Binding Factor 1
LMNA	Lamin A/C

MMP	Matrix Metalloproteinase
MVD	Mitral Valve Disease
NCOA3	Nuclear Receptor Coactivator 3
N.S.	Not Significant
NIH-NCI	National Institutes of Health – National Cancer Institute
NKD2	Naked Cuticle Homolog 2
OIMA	Online Mendelian Inheritance in Animals
PCR	Polymerase Chain Reaction
PDE3B	Phosphodiesterase 3B
PDK4	Pyruvate Dehydrogenase Kinase 4
qRT-PCR	quantitative Reverse Transcriptase PCR
RNA	Ribonucleic Acid
RNAseq	RNA sequencing
SD	Standard Deviation
SEER	Surveillance, Epidemiology, Evidence and End Results
SEM	Standard Error of the Mean
SLC2A1	Solute Carrier Family 2 Member 1
SNP	Single Nucleotide Polymorphism
STRN	Striatin
TP53	Tumour Protein p53
UCSC	University of California, Santa Cruz
WHO	World Health Organisation

Chapter 1 Introduction: Comparative models of disease

1.1. Dogs as models of health and disease

1.1.1. Global health burden

Non-communicable diseases such as heart disease, cancer, and diabetes are the leading causes of morbidity and mortality in people worldwide (WHO, 2011). It is predicted that the percentage of deaths from non-communicable diseases will rise by 15% between 2010 and 2020 from 63% in 2008 (WHO, 2011). With this increase in disease burden there is a need for improved disease prevention strategies, diagnosis, and treatment.

Prevention of disease requires a thorough understanding of the underlying causes of disease. Where the causes of disease are understood it can allow for modifications in diet, behaviour, and/or preventative medicine to be prescribed, or risk-reducing surgery to be undertaken where appropriate (Banks et al., 2015, Domchek et al., 2010, McFadden et al., 2015). The underlying causes of non-communicable diseases are varied with a wide range of environmental and genetic factors contributing to disease (Hall et al., 1990, Parkin, 2011b, Parkin, 2011a, Parkin, 2011c, Parkin, 2011d, Wooster et al., 1995). Some risk factors for disease are well substantiated. Tobacco accounts for the majority of lung cancer cases, and mutations in *BRCA* genes confer a large increased risk of developing breast and ovarian cancer (Antoniou et al., 2003, Parkin, 2011a). Although there are examples of a single factor as the major contributor to disease, these typically are the exception to the rule. In most cases a combination of interacting factors contribute to disease risk, initiation, and progression (Chu et al., 2012, Nickels et al., 2013, Sharafeldin et al., 2015, Song et al., 2014).

Diagnosis of disease is informed by patient symptoms, family history, medical testing, and in some cases genetic testing (Cooper et al., 2009, Mant et al., 2007, Porto et al., 2015). With greater understanding of disease progression, these aspects can be more accurately assessed and give earlier accurate diagnoses (Andrae et al., 2012). Early diagnosis can enable early treatment,

which can result in improved outcomes compared to patients diagnosed later in the disease course (Andrae et al., 2012, Nordestgaard et al., 2013, Rebbeck et al., 2004). For example screening for disease prior to the onset of symptoms has been shown to successfully improve outcomes for cervical cancer (Andrae et al., 2012). However, screening all people for all diseases in this way is not practical and in some cases is not recommended because of the lack of specificity of the test, but screening can be useful for some individuals (Moyer, 2012). Screening asymptomatic individuals can be recommended when there is an additional reason to suspect that an individual may develop disease, such as family history of the disease or a genetic test result indicating susceptibility to disease (George et al., 2015, Ranthe et al., 2015). A positive genetic test result often does not fully predict disease development, merely that the individual has a genetic pre-disposition to developing the disease (Antoniou et al., 2003). Thus a positive genetic test result does not always result in direct medical intervention, it can lead to increased awareness of the disease and enrolment of the individual on a health monitoring programme (Nordestgaard et al., 2013, Ranthe et al., 2015).

Treatment of disease varies depending on the disease. The ultimate aim of treatment is to cure disease, but this is not currently always possible (Andrae et al., 2012, DeSantis et al., 2014). When a cure is not available, treatment of disease is aimed at reducing the impact of the disease, extending lifespan, and increasing quality of life (Nordestgaard et al., 2013). Developing new treatments is expensive and time consuming, only 4.1% of potential new compounds progress from preclinical discovery to ultimately becoming available for patients, and it takes on average 13.5 years for this process to be completed (Calcoen et al., 2015, Paul et al., 2010). Insights from the underlying genetic mechanisms of disease have the potential to lead to more targeted treatments in a shorter period of time (Nelson et al., 2015).

1.1.2. Animal models of disease

Current understanding of disease processes and treatments is based on studying affected individuals compared to unaffected individuals, along with the use of animal models of disease, cell lines, and computer simulations (Al-

Mamun et al., 2016, Betz et al., 2015, Brown et al., 2015, Eckstein et al., 2013, Lee et al., 2015, Mariani et al., 2013, Simoni et al., 2002). While these methods have improved prevention, diagnosis, and treatment of a range of diseases, there are diseases where cure rates and survival times have not improved in decades (Perrin et al., 2009, Reddy et al., 2015, Siegel et al., 2015, Wang et al., 2013b). For diseases such as these, including osteosarcoma for example, there is an urgent need for improved treatments (Reddy et al., 2015). This will only be possible with a greater understanding of the underlying disease biology. Essential aspects to increasing this understanding are high quality disease models, including animal models.

Animal models are used to test hypotheses prior to translation into human studies. These hypotheses can include testing whether a factor such as exposure to a chemical or other environmental factor causes disease, whether a treatment intervention is effective, or whether a genetic locus is involved in disease development (Druker et al., 1996, Legendre, 2013, Martins-Green et al., 2014, Matsushita et al., 2015, Stanga et al., 2002). The advantage of animal models under laboratory conditions is that everything apart from the aspect of disease development or treatment that is being tested can be tightly controlled with both cases and control groups exposed to the same environmental conditions (Festing and Altman, 2002). Conversely laboratory animal models often do not accurately reflect natural disease initiation and development. Often laboratory animal models only replicate a small component of the disease and confounding factors such as co-morbidities are not accounted for (Begley and Ellis, 2012, Denayer et al., 2014, van der Worp et al., 2010). Mechanical or chemical induction of disease is often used in laboratory animals, but it does not typically reflect natural disease progression as it is performed over short time scales of hours to weeks, whereas human disease usually progresses over many years (Davies et al., 1991, Gu et al., 2006, Sandusky et al., 1981, Saito et al., 2015). Genetically modified animal models target the alteration of specific genes and with the use of gene editing technology such as CRISPR/Cas this has been greatly refined (Cong et al., 2013, Jinek et al., 2012, Yang et al., 2014a). Despite the integration of specific mutations the resulting phenotypes do not necessarily recapitulate the human phenotype. For example genetically

modified mice with cystic fibrosis do not develop the characteristic lung pathology, while a pig model does (Grubb and Boucher, 1999, Hoegger et al., 2014). In spite of this, the use of animal models to advance human medicine is valuable. For example historically the use of dogs has advanced surgical techniques such as heart transplant surgery and blood transfusions, and advanced medical treatments such as the discovery of insulin (Nathoo et al., 2009, Richmond, 2006, Simoni et al., 2002). Examples of recent advances include administering gene therapy to human patients with heart failure following success in animal models, and developing new surgical techniques such as near-infrared fluorescence imaging (Atallah et al., 2014, Bredell, 2010, Gleysteen et al., 2008, Greenberg et al., 2016). Although the use of animal models in developing new treatments and understanding the basis of disease is essential, caution must be used to ensure that phenotypes are representative of the disease of interest (Houser et al., 2012). In addition there are ethical concerns when performing experiments on animals and as such where possible the use of animals is replaced, reduced, and refined to maximise the benefit of the experiment whilst minimising harm (Curzer et al., 2016).

Natural models of disease allow researchers access to additional cases of disease without inducing disease and causing additional suffering because the animals involved develop disease irrespective of involvement in a study. As such an additional and relevant resource for investigating health and disease is the companion animal population, within which dogs in particular are useful. The canine population overall is genetically heterogeneous, yet breeds are comparatively homogeneous which enhances their value as genetic models of disease (Parker et al., 2004). A further advantage of utilising specific dog breeds is that some breeds are at increased risk of developing particular diseases which make them natural models of the equivalent human disease (Basso et al., 2004, Davidson et al., 2007, Egenvall et al., 2006, Egenvall et al., 2007, Zangerl et al., 2006). Many canine disease phenotypes can be closely matched to human disease phenotypes with similar disease progression, pathology, treatment options, and prognosis (Lenffer et al., 2006, OMIA, 2016, Tsai et al., 2007). Indeed there are currently 383 potential models for human disease listed in OMIA (Online Mendelian Inheritance in Animals) for dogs,

greater than any other species (Lenffer et al., 2006, OMIA, 2016). Dogs are typically treated as family members and so inhabit the same environment as their owners with the associated exposure to the same potential environmental toxins, including for example air pollution (Calderón-Garcidueñas et al., 2008). Pet dogs also frequently benefit from high quality medical care, such that illnesses are detected and treated promptly, similar in a way to the human population (Ostrander et al., 2000). These characteristics of the canine population make it a valuable resource as a model of human disease. Examples of diseases with homologies in humans and dogs include diabetes, cardiomyopathies, cancers, and eye diseases (Basso et al., 2004, Fosmire et al., 2007, Lakey et al., 1998, OMIA, 2016, Zangerl et al., 2006).

1.1.3. Genetic basis of disease

The development and progression of common non-communicable diseases such as cancers, heart diseases, and diabetes are influenced by a combination of risk factors. These risk factors include both environmental and genetic effects (Hall et al., 1990, Parkin, 2011b, Parkin, 2011a, Parkin, 2011c, Parkin, 2011d, Wooster et al., 1995). It has been shown in several diseases that interactions between environmental and genetic risk factors are important in the development and progression of disease (Nickels et al., 2013, Song et al., 2014). Environmental risk factors are often modifiable as individuals can make informed choices with regards to lifestyle changes to reduce their risk of developing disease (Blair et al., 1995, Parkin, 2011d, Parkin, 2011b). Currently genetic risk factors are not modifiable, although genome editing technologies may allow this in the future (Maeder and Gersbach, 2016). Despite this, individuals identified as having a high genetic risk of developing a disease are currently able to reduce their risk of developing disease. Options available include making lifestyle changes to reduce other risk factors, increased disease surveillance, and prophylactic medicine and surgery (Blair et al., 1995, Domchek et al., 2010, George et al., 2015, Nordestgaard et al., 2013, Parkin, 2011d, Parkin, 2011b, Ranthe et al., 2015). Increased disease surveillance can allow for early disease detection and associated early treatment which is associated with improved prognosis (Andrae et al., 2012, Nordestgaard et al., 2013). Prophylactic medicine and surgery are options in a limited number of

diseases including surgery for breast and ovarian cancers in *BRCA1* and *BRCA2* mutation carriers, and LDL-cholesterol lowering drugs for those at risk of cardiovascular disease (Li et al., 2016b, McFadden et al., 2015). An additional benefit of knowledge of the underlying genetic cause of disease could lead to targeted treatments such as rectifying the defective gene or drugs targeting affected pathways (Greenberg et al., 2016, Ho et al., 2015).

Genetic loci conferring susceptibility to disease have been identified, and associated genetic tests have been developed for 10485 conditions in people (GTR®, 2016, Rubinstein et al., 2013). Despite this there are still many cases of disease that are thought to have a genetic component for which there are no genetic loci identified (Haas et al., 2015, Oliveira et al., 2015, Ullemar et al., 2016). Identification of susceptibility loci for these diseases has the potential to bring improvements in diagnosis and may lead to improved treatments in line with diseases which already have genetic loci associated with them (Andrae et al., 2012, Greenberg et al., 2016, Ho et al., 2015, Kaback, 2000, Li et al., 2016b, Nordestgaard et al., 2013).

1.1.3.1. Wnt signalling

A key pathway involved in the development of many diseases is the Wnt signalling pathway. The Wnt signalling pathway is conserved across species (Komiya and Habas, 2008). It has a wide range of effects in both normal development and disease processes, including both cancers and heart disease (Nagaraj et al., 2015, Liu et al., 2013, Hou et al., 2016, Goliasch et al., 2012, Mani et al., 2007).

The Wnt signalling pathway is initiated extracellularly when Wnt proteins bind to transmembrane proteins from the Frizzled receptor family (Bhanot et al., 1996, Dann et al., 2001). Activation of a Frizzled receptor results in a signal being transmitted to Dishevelled within the cytoplasm (Habas and Dawid, 2005, Zeng et al., 2008). It is at this point in the signalling cascade that Wnt signalling splits into separate signalling cascades – canonical and non-canonical (Veeman et al., 2003).

The canonical cascade is β-catenin dependent, while the non-canonical cascade is β-catenin independent (Habas and Dawid, 2005). Canonical signalling disrupts the normal proteasome-mediated degradation of β-catenin within the cytoplasm (Logan and Nusse, 2004, Topol et al., 2003). This allows β-catenin to accumulate within the cytoplasm and nucleus, whereupon within the nucleus it interacts with transcription factors affecting gene transcription (Logan and Nusse, 2004).

The non-canonical cascade is further split into the Wnt/Ca^{2+} pathway and the Planar Cell Polarity (PCP) pathway (Habas and Dawid, 2005).The Wnt/Ca^{2+} leads to an increase in intra-cellular Ca^{2+} (Kim et al., 2016b, Kühl et al., 2000). This pathway has been shown to be important in cell movement and migration, and thus is important during gastrulation and embryo development (Komiya and Habas, 2008). The Wnt/PCP pathway maintains cellular and tissue polarity and has been shown to have roles in the maintenance of tight junctions in brain endothelial cells, neural crest migration and cancer progression (Artus et al., 2014, Luga and Wrana, 2013, Mayor and Theveneau, 2014, Wang, 2009).

In addition to the binding of Wnt proteins to the transmembrane Frizzled receptors there are many molecules that interact with the pathway. These include co-receptors and repressors acting when Wnt proteins initiate the Wnt signalling pathway (Zeng et al., 2008, Lu et al., 2004, Hall et al., 2010). In addition there are interactions throughout the pathway where modifications can be made to the signalling pathway (Topol et al., 2003, Nakamura et al., 1998, Kim et al., 2013c). Wnt signalling has been shown to be important in the development of a range of diseases (Nagaraj et al., 2015, Liu et al., 2013, Hou et al., 2016, Goliasch et al., 2012, Mani et al., 2007). Due to this it is possible that genes relating to this pathway could be implicated in additional non-communicable diseases.

1.1.4. Genetic basis of canine disease

Animal models of disease play crucial roles in identifying genetic loci associated with disease (Wang and Paigen, 2005, Zangerl et al., 2006, Zhang et al., 1994).

This is facilitated by the conservation of many fundamental biological pathways between species (Guruharsha et al., 2012, Le et al., 2011, Loenarz et al., 2011). The pedigree pet dog population is of particular interest as a genetic model of disease. Each breed of dog is a closed population and ancestry can typically be traced for many generations, often to the founding members of the breed (Calboli et al., 2008, Jansson and Laikre, 2014, Parker et al., 2004). This facilitates understanding the mode of inheritance of traits and diseases, and also restricts the amount of genetic diversity within a breed (Calboli et al., 2008, Davidson et al., 2007, Distl et al., 2007, Willet et al., 2015). Founder effects and subsequent inbreeding within pedigree dog breeds have led to differing allele frequencies between breeds and some breeds more prone to developing particular conditions than others (Marsden et al., 2016, Olsson et al., 2014, Parker et al., 2004). This makes breeds with homologs of human conditions ideal for identifying potential genetic loci associated with disease for both canine and human benefit (Decker et al., 2015, Zangerl et al., 2006).

The tendency for particular breeds of dog to develop particular diseases has the potential to benefit human medicine, but it is also a clinical veterinary issue in its own right (Egenvall et al., 2006, Egenvall et al., 2007, Pelander et al., 2015). As outlined earlier, pet dogs are often treated as members of the family (Kanat-Maymon et al., 2016). As such their health and wellbeing is supported by advanced veterinary care, often funded by veterinary medical insurance (Pelander et al., 2015). Yet, despite known pre-dispositions to diseases in the majority of UK kennel club registered breeds, there are currently only 93 disease associated variants identified and only 61 genetic tests commercially available across all breeds (Donner et al., 2016, Farrell et al., 2015, The Kennel Club UK, 2016b). This is a lot less than available in human genetic testing despite close similarity between many diseases affecting dogs and humans (Lenffer et al., 2006, OMIA, 2016, Tsai et al., 2007). Many tools have been utilised when identifying genetic variants associated with human diseases, these include genome wide association studies, candidate gene studies, whole exome sequencing, and whole genome sequencing (Butler et al., 2011, Rivas et al., 2011, Saunders et al., 2012, Yang et al., 2013). These tools have also been utilised to some extent in some canine studies, but these approaches are

often restrictively expensive for companion animal studies with limited funding available (Mausberg et al., 2011, Philipp et al., 2012). The cost of these methods is delaying their clinical and research application in evaluating the genetic basis of canine diseases.

1.1.5. Associations between canine diseases

The existence of veterinary insurance-derived databases has enabled determination of disease association studies. One example exploited the Swedish insured dog population where there are surprising associations between the incidence of heart disease and bone tumours within breeds. There were more than 330 breed designations within the dataset, but of the 12 breeds with the highest incidence of bone tumours, 8 also had the highest incidence of heart disease (Figure 1.1) (Egenvall et al., 2006, Egenvall et al., 2007). Additional studies have utilised the same population to examine incidence of kidney disease and diabetes. While some of the affected breeds are the same there are fewer similarities in the most affected breeds (Figure 1.1) (Fall et al., 2007, Pelander et al., 2015). In the diabetes study only two of the 12 breeds most prevalent for diabetes are within the 12 most prevalent breeds for kidney disease, bone tumours, and heart disease combined (Figure 1.1) (Egenvall et al., 2006, Egenvall et al., 2007, Fall et al., 2007, Pelander et al., 2015). It is a similar case for kidney disease with only three breeds appearing within an additional study's list for the highest incidence (Figure 1.1) (Egenvall et al., 2006, Egenvall et al., 2007, Fall et al., 2007, Pelander et al., 2015). This suggests that there could be a biological relationship between the development of heart disease and bone tumours in the breeds with high incidence. Irish wolfhounds in particular have the highest incidence of both heart disease and bone tumours in the insured Swedish dog population (Figure 1.1) (Egenvall et al., 2006, Egenvall et al., 2007).

Incidence ranking	Diabetes	Kidney disease	Bone tumour	Heart disease
1	Australian Terrier	Bernese Mountain Dog	Irish Wolfhound	Irish Wolfhound
2	Samoyed	Miniature Schnauzer	St. Bernard	Cavalier King Charles Spaniel
3	Swedish Lapphund	Boxer	Leonberger	Great Dane
4	Swedish Elkhound	Yorkshire Terrier	Great Dane	St Bernard
5	Border Collie	Dalmatian	Rottweiler	Newfoundland
6	Finnish Hound	Shetland Sheepdog	Flat-coated Retriever	Leonberger
7	Drever	Fox Terrier	Greyhound	Dobermann
8	West Highland White Terrier	Shih Tzu	Hovawart	Finnish Hound
9	Hamilton Hound	Soft-coated Wheaten Terrier	Bernese Mountain Dog	Boxer
10	Beagle	Cairn Terrier	Doberman	Giant Schnauzer
11	Poodle (Miniature and Toy)	Giant Schnauzer	Newfoundland	English Cocker Spaniel
12	Rottweiler	Bearded Collie	Boxer	Flat-Coated Retriever

Figure 1.1. The 12 breeds with the highest incidence of diabetes, kidney disease, bone tumour, and heart disease adapted from (Egenvall et al., 2006, Egenvall et al., 2007, Fall et al., 2007, Pelander et al., 2015). Lines indicate where breeds appear on multiple lists. Red = Bone tumour and Heart disease, Blue = Kidney disease and Bone tumour, Green= Kidney disease and Heart disease, Purple = Diabetes and Bone tumour, Orange = Diabetes and Heart disease.

Bone tumours and heart disease appear to be unrelated conditions with no obvious mechanistic link. However, a common association in both the heart disease and bone tumour incidence studies is that of sex. Males of all breeds appear to develop bone cancer and heart disease at a higher rate than females, before the age of 10 years (Egenvall et al., 2006, Egenvall et al., 2007). There is some evidence that females may develop both conditions just as frequently, it tends to occur later in life than males (Phillips et al., 2007, Wess et al., 2010). Within the heart disease diagnosis study the most prevalent specific diagnosis is dilated cardiomyopathy (DCM) (Egenvall et al., 2006). It is not possible to know the specific type of bone tumours involved in the analysis by Egenvall *et al.* (2007), however, previous work has shown that the most common type of canine bone tumour is osteosarcoma, accounting for up to 85% of bone tumours (Anfinsen et al., 2011, Brodey et al., 1963, Brodey and Riser, 1969, Liu et al., 1977). Due to the fact that the most commonly affected breeds for both heart disease and bone tumours are so frequently the same (Figure 1.1) it is plausible that there may be aspects of disease development shared between diseases and across breeds. For this reason, this thesis has focussed on dog breeds known to have increased risk of both osteosarcoma and DCM.

1.2. Endocrine signalling and disease

1.2.1. Endocrine signalling

For many diseases there is a sex disparity in incidence where the disease is either more common or occurs at an earlier age in one sex (Edgren et al., 2012, Kramer et al., 2015, Safford et al., 2012). In people and dogs both DCM and osteosarcoma have been shown to have a difference in disease progression between the sexes, with males typically affected more frequently and at younger ages (Bagger et al., 1984, Doesch et al., 2014, Egenvall et al., 2007, Lin et al., 2015, Martin et al., 2010, Mirabello et al., 2009b, Truszkowska et al., 2015, Wess et al., 2010). These differences may be due to endocrine differences between males and females (Dabbs, 1990, Torjesen and Sandnes, 2004). Some disease processes such as Parkinson's disease, heart disease, and cancers, have been shown to have a relationship with hormone secretion and response (Benn et al., 2015, English et al., 2000, Hsing et al., 2000, Nitkowska et al., 2015, Rebbeck et al., 1999). The secretion of, and response to, hormones is dependent on a wide range of variables including physiological and psychological stress, diet, age, and sex (Ishii et al., 2016, Koelsch et al., 2016, Kushnir et al., 2010, Maersk et al., 2012, Spanakis et al., 2016, Stephens et al., 2016). In addition genetic factors have been shown to contribute to the secretion and response to hormones (Ibanez et al., 2003, Mongan et al., 2003, Vasseur et al., 2002).

1.2.2. Endocrine differences between sexes

While the secretion and response to hormones is different from individual to individual there are differences in the production of the sex steroid hormones, the androgens and estrogens, between the sexes (Bidlingmaier et al., 1973, Kushnir et al., 2010, Nelson et al., 2004). Males typically have higher concentrations of circulating androgens than females, whereas females typically have higher levels of circulating estrogens than males (Bidlingmaier et al., 1973, Kushnir et al., 2010, Nelson et al., 2004). Physiological androgens include androstenediol, dehydroepiandrosterone, testosterone, dihydrotestosterone, and androstenedione (Kushnir et al., 2010, Morales et al., 1998). There are no differences between the sexes in the concentrations of dehydroepiandrosterone, but adult males usually have higher concentrations of

androstenediol, testosterone, and dihydrotestosterone, and lower concentrations of androstenedione as compared to premenopausal adult females (Dabbs, 1990, Kushnir et al., 2010, Morales et al., 1998, Rosenfield and Otto, 1972). The physiological estrogens include estradiol, estrone, and estriol (Kern Jr et al., 1981, Nelson et al., 2004, Romani et al., 2003). Estradiol and estrone concentrations remain similar throughout adult male life, females, however, go through the menopause following which hormone levels change (Nelson et al., 2004, Sowers et al., 2013). Pre-menopausal women have higher concentrations of estradiol and estrone than males, but in post-menopausal women estrogen concentrations reduce to a level lower than in men (Nelson et al., 2004). Estriol is found at much lower concentrations than estradiol and estrone except during pregnancy when concentrations increase to within the same range as estradiol and estrone (Kern Jr et al., 1981, Romani et al., 2003). Both androgens and estrogens are derived from cholesterol, with androgens intermediary products in the synthesis of estrogens (Cui et al., 2013, Kanehisa et al., 2016) (Figure 1.2). The production of androgens from cholesterol initially occurs in the gonads and adrenal cortex, but subsequent conversion to other androgens and estrogens can then occur in peripheral tissues such as adipose tissue (Udhane and Flück, 2016).

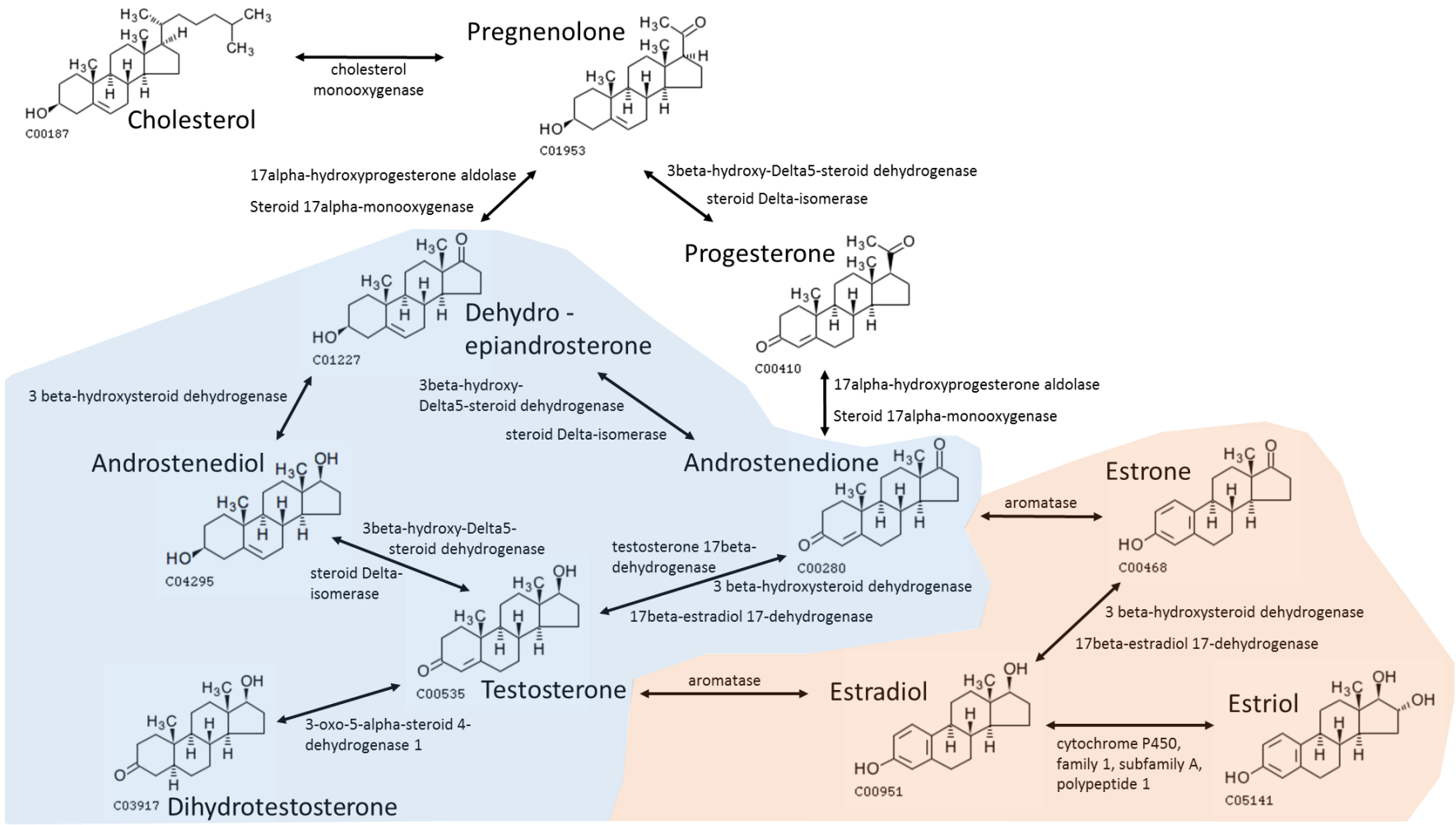


Figure 1.2. Steroid hormone biosynthetic pathway adapted from KEGG pathway maps (Kanehisa et al., 2016). Androgens are within the blue shaded area and estrogens are within the orange shaded area.

Androgens and estrogens exert their biological effects as agonists of the ligand-dependent super family of transcription factors (Green et al., 1986, Greene et al., 1986, Lu et al., 2006, Mosselman et al., 1996). Activation of the androgen receptor (AR) and estrogen receptors α and β (ERα and ERβ) affects subsequent downstream gene transcription (Björnström and Sjöberg, 2005, Lu et al., 2006, Wang et al., 2007). Males and females both usually have functional AR, ERα, and ERβ, but activation is dependent on the binding of androgens and estrogens, thus the biological responses differ between males and females (Dart et al., 2013, Heine et al., 2000). Mutations in the *AR*, *ERα*, and *ERβ* genes can alter the sensitivity of the receptors to their ligands (Fuqua et al., 2000, Gottlieb et al., 2012, Herynk and Fuqua, 2004, Infante et al., 2016). Some mutations within the *AR* gene have been shown to cause complete androgen insensitivity where genetically 46XY males appear to be phenotypically female (Batch et al., 1992, Brown et al., 1988, Infante et al., 2016, Mongan et al., 2015). Other mutations in the *AR* gene can cause partial androgen insensitivity with a range of clinical presentations including gynecomastia, genital under-masculinization, and hypospadias (Bevan et al., 1996, Bevan et al., 1997, Evans et al., 1997). Somatic mutations in both the *ERα* and *ERβ* genes are rare but have been associated with increased and decreased sensitivity to estrogen (Fuqua et al., 2000, Quaynor et al., 2013, Smith et al., 1994). Increased sensitivity to estrogen has been demonstrated to be a common factor in the development of premalignant breast lesions (Fuqua et al., 2000). Naturally occurring mutations associated with estrogen insensitivity are much rarer but have been associated with amenorrhea and absent breast development in a woman, and genu valgum “knock-knee” and unfused epiphyses in a man (Quaynor et al., 2013, Smith et al., 1994).

The response to androgens and estrogens is dependent on their binding to AR, ERα, and ERβ (Björnström and Sjöberg, 2005, Lu et al., 2006, Wang et al., 2007). AR, ERα, and ERβ are transcription factors and thus activation affects downstream gene transcription (Björnström and Sjöberg, 2005, Lu et al., 2006, Wang et al., 2007). Some androgens and estrogens are stronger agonists of the receptors than others and thus the intensity of the response is different depending on the agonist present (Blair et al., 2000, Fang et al., 2003, Kuiper

et al., 1997). Variability in the binding of androgens and estrogens to their receptors is also dependent on the expression levels of epigenetic coregulators and in addition the receptors themselves have variation in their ability to bind to their ligands (Boorjian et al., 2009, Chamberlain et al., 1994, Gnanapragasam et al., 2001, Kazemi-Esfarjani et al., 1995, Ngan et al., 2003, Yepuru et al., 2013). The variation in the ability of the receptors to bind to their ligands has been associated with a range of diseases and disorders. There has been greater variation identified in the ability of AR to bind to its ligands than ERα, and ERβ, however a variant in the *ERα* gene associated with increased sensitivity to estrogens is commonly found in human premalignant breast lesions (Fuqua et al., 2000). Within the *AR* gene there is a polymorphic polyglutamine tract, with individuals with longer polyglutamine tracts less sensitive to androgens and individuals with shorter polyglutamine tracts more sensitive to androgens (Chamberlain et al., 1994, Kazemi-Esfarjani et al., 1995). Variation in the length of this polymorphic polyglutamine tract has been associated with a range of human diseases including cancers, Kennedy's disease, and ovarian hyperandrogenism (Giovannucci et al., 1997, Hsing et al., 2000, Ibanez et al., 2003, Rebbeck et al., 1999, Spada et al., 1991). The canine *AR* gene contains two polyglutamine tracts and variation in the length of these tracts has been associated with prostate cancer in a variety of breeds and aggression in male Japanese Akita Inus (Konno et al., 2011, Lai et al., 2008).

1.2.3. Androgens and estrogens associated with disease

Androgens and estrogens have been shown to contribute to a range of diseases and disorders including cancers, ovarian hyperandrogenism, Kennedy's disease, osteoporosis, and heart disease (English et al., 2000, Ettinger et al., 1998, Hsing et al., 2000, Ibanez et al., 2003, Nguyen et al., 2015, Rebbeck et al., 1999). In some instances the amount of hormones have been shown to contribute to the disease process, whereas in other instances it is the response to hormones that has been shown to be significant (Daniell et al., 2000, English et al., 2000, Ettinger et al., 1998, Giovannucci et al., 1997, Hsing et al., 2000, Ibanez et al., 2003, Jackson et al., 1992, Laughlin et al., 2010, Nguyen et al., 2015, Rebbeck et al., 1999, Spada et al., 1991, Wu and Eckardstein, 2003). Coronary artery disease (CAD) is an example where

hormones have been associated with development of disease. Males with low testosterone have been shown to have an increased risk of developing CAD compared to men with high testosterone (English et al., 2000, Wu and Eckardstein, 2003). In women >50 years old individuals with particularly high or low testosterone have an increased risk of CAD compared to individuals with intermediary testosterone (Laughlin et al., 2010). The development of osteoporosis in both men and women is associated with alterations in the amount of hormones. In both women and men osteoporosis development is associated with low estradiol, and in men low testosterone is also associated with the development of osteoporosis (Daniell et al., 2000, Ettinger et al., 1998, Jackson et al., 1992, Nguyen et al., 2015).

1.2.4. Androgen Receptor Signalling

The androgen receptor protein consists of four domains; a N-terminal domain, a DNA-binding domain, a hinge region, and a ligand-binding domain (Lonergan and Tindall, 2011) (Figure 1.3). The ligand-binding domain is responsible for the binding of ligands to the receptor and initiating activation of the AR complex (Matias et al., 2000, Jaaskelainen et al., 2006). When a ligand binds to the ligand-binding domain it induces a conformational change in the AR protein (Reid et al., 2003). The hinge region of the AR then interacts with a cytoskeletal protein, Filamin-A (FlnA) to facilitate the translocation of AR into the nucleus (Ozanne et al., 2000, Lonergan and Tindall, 2011). Following activation and translocation into the nucleus the DNA-binding domain is responsible for tethering the AR to the promotor/enhancer regions of target genes affecting transcription (Reid et al., 2003). The conformational changes that occurred from ligand binding results in a docking site for co-regulators and a region of the N-terminal domain inducing further conformational changes affecting AR activity (Buchanan et al., 2004, Berrevoets et al., 1998). Co-regulators include co-activators and co-repressors, where co-activators increase transcriptional activity of AR while co-repressors reduce it (Culig et al., 2004, Wang et al., 2005a). A simplified diagram of this AR signalling pathway is shown in Figure 1.4.

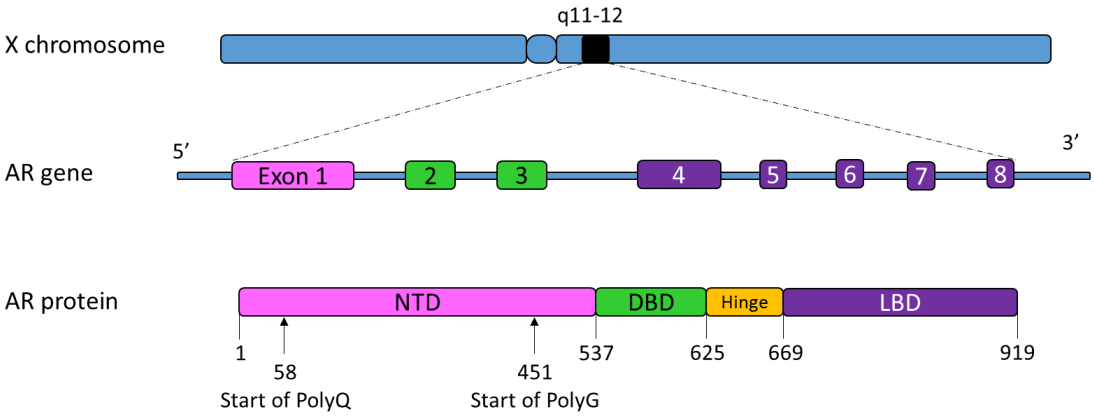


Figure 1.3. The location of the AR gene on the X-chromosome, its exons, and domains. Exons are colour coded to show which domain they code for. Where the Polygluamine (PolyQ) and Polyglycine (PolyG) tracts begin in the protein are indicated. Modified from (Lonergan and Tindall, 2011).

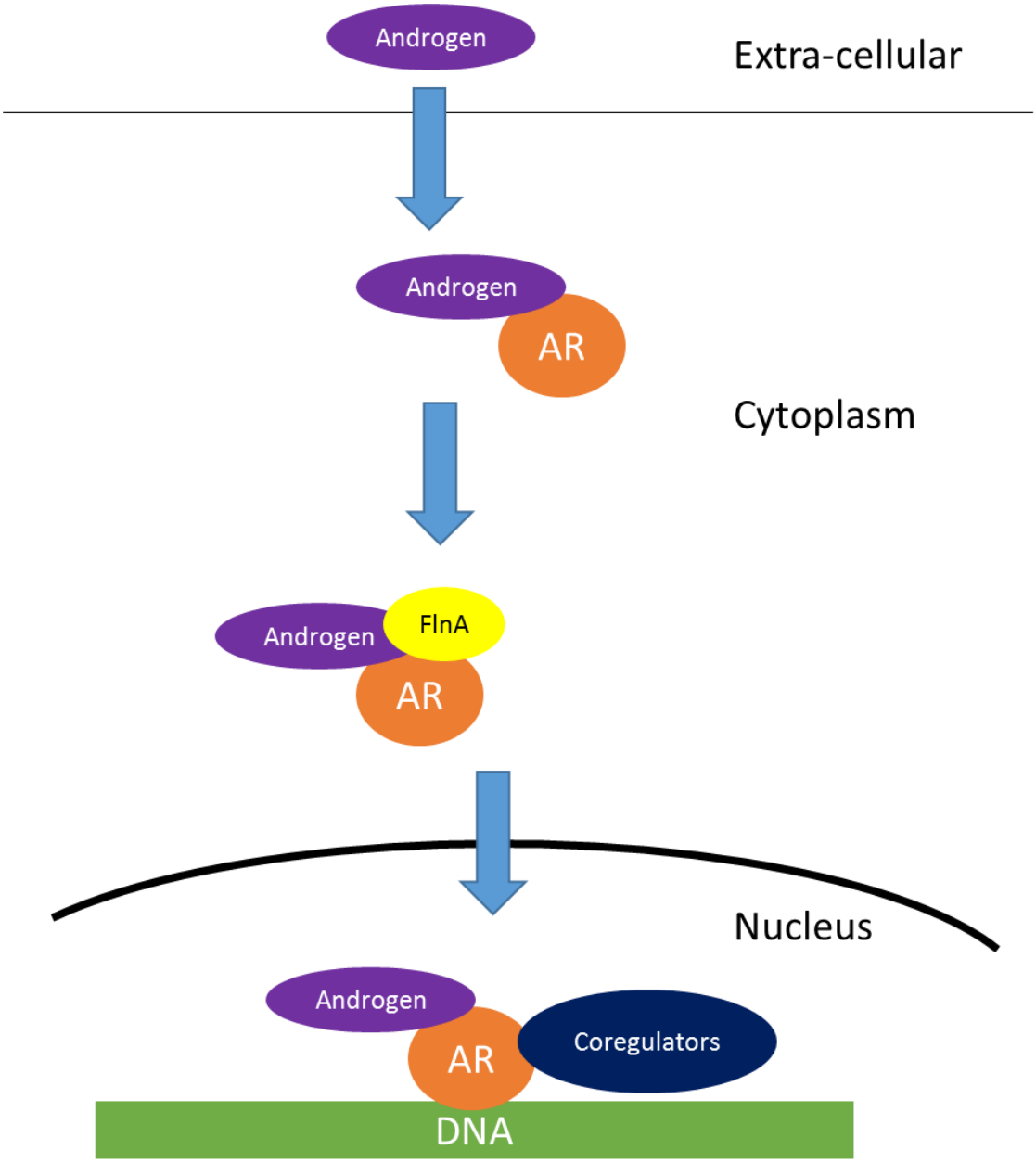


Figure 1.4. Simplified diagram of the Androgen Receptor (AR) signalling pathway. Androgens enter the cell and bind to the ligand-binding domain of the AR, this induces conformational changes allowing the hinge region to interact with the cytoskeletal protein Filamin-A (FlnA) which facilitates the translocation of the AR into the nucleus where the DNA binding domain tethers the AR to promotor/enhancer regions of target genes. The conformational changes that occurred from ligand binding results in a docking site for co-regulators and a region of the N-terminal domain inducing further conformational changes affecting AR activity. Modified from (Lonergan and Tindall, 2011).

Variations in the AR protein lead to differences in activity with mutations found throughout the AR gene (Gottlieb et al., 2012). For example mutations affecting the ligand binding domain can alter how the AR binds to androgens, thus altering activation of the AR (Matias et al., 2000, Bolzon et al., 2016, Uo et al., 2016), while mutations within the N-terminal domain have been shown to affect the recruitment of co-regulators to the AR complex thus affecting transcription (Buchanan et al., 2004). A particularly variable part of the *AR* gene encodes a polyglutamine tract within the N-terminal domain that contributes to the activity of the AR protein via interaction with co-regulators (Buchanan et al., 2004). Polyglutamine tracts are often hypervariable with functional implications for protein-protein interactions (Schaefer et al., 2012). The number of repeats within the AR polyglutamine tract varies between individuals and populations (Buchanan et al., 2004). Individuals with repeat numbers the ends of the spectrum typically have syndromes such as Kennedy's disease and ovarian hyperandrogenism (Ibanez et al., 2003, Spada et al., 1991). More polyglutamine repeats lead to a reduction in the ability to recruit co-regulators thus reducing the effects on downstream transcription, whilst the opposite is the case fewer repeats increases the recruitment of co-regulators (Buchanan et al., 2004).

In addition to variation in the number of polygulatamine repeats within the AR, variation within co-regulators also has an effect on downstream transcription (Heinlein and Chang, 2002). NCOA3 is one such co-regulator, it is a co-activator and it has within it a polyglutamine tract (Mongan et al., 2003). Due to the implications for protein-protein interactions, variability within the polyglutamine tract could influence its interaction with AR (Schaefer et al., 2012). Indeed there is evidence for association between the length of the NCOA3 polyglutamine tract and AR activity (Mongan et al., 2003).

The association of androgens, or the response to androgens, has been implicated in both heart disease and cancers (English et al., 2000, Hsing et al., 2000, Laughlin et al., 2010, Rebbeck et al., 1999, Wu and Eckardstein, 2003). Due to this potential shared pathway the response to androgens due to variation in the polyglutamine tracts of AR and NCOA3 was considered in this

thesis as a potential aspect of both canine DCM and osteosarcoma development. Further evidence for a possible effect of androgens in DCM and osteosarcoma development is that both diseases have a sex difference in development in both humans and dogs, which could be explained by differing concentrations of androgens between males and females (Egenvall et al., 2007, Mirabello et al., 2009b). If androgens, or the response to androgens, are implicated in canine disease this could be a potential aspect to be considered in human DCM and osteosarcoma development.

1.3. Fatty acid profiles and disease

Many diseases involve inflammatory responses either as a reaction to disease or in the initiation of the disease process; although inflammation itself is not always detrimental, for instance it is an important aspect of wound repair (Danesh et al., 2000, Gurtner et al., 2008, Harris, 2009, Mohler et al., 2001). Elevated markers of inflammation are frequently detected in heart failure and cancers although this could be due to the response to disease, or the underlying cause of disease (Aukrust et al., 1998, Il'yasova et al., 2005, Leyva et al., 1998, Pankuweit et al., 2013, Shiels et al., 2013).

Important contributors to the inflammatory response are fatty acid-derived eicosanoids (Bagga et al., 2003, Harris, 2009, Vane, 1971). Fatty acids consist of a carboxylic acid with a hydrocarbon chain tail (Fahy et al., 2005). The length of the hydrocarbon chain varies between fatty acids, as does the presence or absence of double bonds between the carbon atoms and their location (Fahy et al., 2005). In addition to being the precursors for eicosanoids and other metabolites, fatty acids are major substrates for energy production, and are essential components of the cell membrane (Fahy et al., 2005, Kröger et al., 2014, Wolters et al., 2014).

Fatty acids can be synthesised into other fatty acids along specific pathways by elongation and desaturation enzymes (Kanehisa et al., 2016, Vessby et al., 2013, Wang et al., 2005b). In mammals the fatty acids are obtained from the diet prior to metabolism or incorporation as components of cells (Farvid et al., 2014, Meyer et al., 2003, Burns et al., 1979, Kröger et al., 2014). There are two

major groups of fatty acids: ω-6 fatty acids are primarily obtained from fats and oils, and ω-3 fatty acids which are obtained primarily from fish and seafood products (Meyer et al., 2003). Mammals are not naturally able to convert between the two major pathways and therefore must obtain precursors of both ω-6 and ω-3 fatty acids from the diet (Kang et al., 2004).

The ω-6 fatty acid arachidonic acid is a precursor of the most important pro-inflammatory eicosanoids, while the ω-3 fatty acid derivatives, eicosapentaenoic acid and docosahexaenoic acid metabolites are considered less inflammatory (Bagga et al., 2003). Arachidonic acid is released from cell membranes by phospholipase A_2 enzymes in response to pro-inflammatory stimuli including (Bonventre et al., 1997, Burch et al., 1986, Lee et al., 2010, Xu et al., 2003). Free arachidonic acid is then available for conversion to eicosanoids by cyclooxygenase, lipoxygenase and cytochrome P450 enzymes (McAdam et al., 1999, Morrison and Pascoe, 1981, Oliw et al., 1981, Palmer et al., 1980). These enzymes are also responsible for the conversion of other fatty acids to their metabolites resulting in rate limiting competition (Bagga et al., 2003). There is some evidence that by increasing the amount of ω-3 fatty acids available to the cyclooxygenase, lipoxygenase, and cytochrome P450 enzymes fewer arachidonic acid derived eicosanoids are produced which could reduce inflammation (Bagga et al., 2003, Todoric et al., 2006).

Due to the difference in the inflammatory response between fatty acid metabolites it is hypothesised that the fatty acid profiles could differ between diseased and healthy individuals. Indeed fatty acid profiles have been shown to be altered in blood and tissues in individuals with a range of conditions compared to unaffected individuals in both humans and dogs. These conditions include heart disease, skin disease, and cancer (Azordegan et al., 2012, Baylin and Campos, 2004, Rupp et al., 2010, Taugbøl et al., 1998). Although it is not known if these differences are contributing to the initiation of disease or are a response to disease processes these differences could be drug targets (Buzzai et al., 2005, Kantor et al., 2000, McAdam et al., 1999, Serisier et al., 2006). In addition there a genes that contribute to fatty acid profile composition and if a particular part of the pathway is shown to be different in individuals with disease

compared to healthy individuals, these could be likely genes for candidate gene studies in the future (Purushotham et al., 2009, Stavinoha et al., 2004).

1.4. Human and Canine Dilated Cardiomyopathy (DCM)

In humans DCM is reported to affect 35.6 in 100,000 people, and is estimated be the third most common inherited type of heart disease (Hershberger et al., 2010a, Raju et al., 2011). DCM is the most common specific heart disease diagnosis within the Swedish insured canine population, following the more general diagnosis of cardiomyopathy, accounting for 10% of the cardiac diagnoses (Egenvall et al., 2006). The aetiology of DCM is complex in that genetic factors, myocardial ischemia, hypertension, toxins, infections, and metabolic defects have been implicated in human disease (McNally et al., 2013).

DCM is characterised by cardiac ventricular chamber enlargement and systolic dysfunction (Guttmann et al., 2014). There are several stages of disease progression, starting with a long asymptomatic period before clinical signs appear (Mahon, 2005, Mausberg et al., 2011). The next stage is characterised by no reported outward clinical signs and the individual usually appears to be healthy, but morphological and cardiovascular electrical changes can be observed (Baig et al., 1998, Brownlie and Cobb, 1999, Mahon, 2005, Mausberg et al., 2011). In the final stage of DCM patients present with clinical signs of heart failure, commonly including cough, depression, dyspnoea, weight loss, and syncope, the individual requires treatment for heart failure, but prognosis is often poor (Mausberg et al., 2011, Tidholm et al., 2001). Ten years after DCM diagnosis roughly 40% of people will have died, although there is a wide variation with some individuals remaining asymptomatic, conversely many individuals suffer from sudden death (Lehrke et al., 2011). Dogs with DCM also have significantly shortened lifespan, mean survival time following diagnosis, usually at the point of developing overt clinical symptoms, is 34 weeks, although, similar to humans, large variations are observed, with some surviving for several months while others only live for a few weeks (Martin et al., 2010, Petric et al., 2002, Vollmar, 2000).

Treatment of DCM is aimed at minimising the effect of heart failure on the patient and delaying disease progression (Jefferies and Towbin, 2010). In people this typically consists of ACE inhibitors and β-blockade, often with a diuretic agent and, in the latter stages of disease progression, inotropic agents are frequently prescribed (Jefferies and Towbin, 2010, Merlo et al., 2014). In dogs treatment usually consists of diuretics, ACE inhibitors, positive inotropes, and other vasodilators (Borgarelli et al., 2001, Dukes-McEwan, 2000). Heart transplants can be used as a last resort in treating heart disease, and while this is a treatment option in people, it is not generally available for canine DCM patients (Taylor et al., 2009).

There are over 50 genes associated with DCM in people, some with multiple mutations, whereas there have only been 10 loci associated with canine DCM (Mausberg et al., 2011, Meurs et al., 2012, Meurs et al., 2013, Philipp et al., 2012, Posafalvi et al., 2012, Schatzberg et al., 1999). We have recently reviewed the genetics of canine and human DCM ((Simpson et al., 2015b), Appendix 1). Since the publication of the review there have been at least 10 additional genes identified with mutations associated or possibly associated with human DCM, but none associated with canine DCM (Al-Yacoub et al., 2016, Gupta et al., 2016, Hussain et al., 2016, Long et al., 2015, Long et al., 2016, Muhammad et al., 2015, Qu et al., 2015, Zhao et al., 2016, Zhou et al., 2016, Zhou et al., 2015). There has, however, been a RNAseq study examining the difference in expression of genes between canine DCM hearts and non-DCM hearts (Friedenberg et al., 2016). In the RNAseq study genes involved in cellular energy metabolism were expressed less in the DCM hearts than the non-DCM hearts (Friedenberg et al., 2016). This is potentially expected as it is known that cellular energy metabolism is different in failing hearts compared to normal hearts (Stanley and Chandler, 2002). The study by Friedenberg *et al.* (2016) included a mixture of affected breeds and did not make use of appropriate breed matched control dogs for the non-DCM control group. Friedenberg *et al.* (2016) are likely to have identified genes that are differentially expressed in canine DCM in general, but the appropriate use of breed matched controls could have yielded important functional pathogenic variation within

specific breeds. Additional work in both humans and dogs is warranted to identify further genetic causes of DCM, but the current canine DCM genetic research is particularly limited.

1.4.1. Atrial fibrillation (AF)

Atrial fibrillation is a cardiac rhythm disturbance (January et al., 2014). In people it can contribute to the risk of having a thromboembolism (stroke) (Wolf et al., 1991, January et al., 2014). AF is not associated with the development of DCM in people, however the presence of AF in people with DCM and heart failure has been shown to negatively influence survival (Unverferth et al., 1984, Wasywich et al., 2010). There are limited reports of an association between AF and a thromboembolism in dogs, however there have been studies that have found an association between AF and DCM development, particularly in IWHs (Brownlie and Cobb, 1999, Vollmar, 2000, Usechak et al., 2012). There is evidence that when IWHs with AF are treated with pimobendan that this increases the time before individuals develop DCM and overt heart failure (Vollmar and Fox, 2016). In this thesis this association between canine AF and DCM will be investigated and if necessary testing for genetic associations will be extended to individuals with AF, not just DCM.

1.4.2. Variants analysed for an association with canine DCM

Within this thesis data on six SNPs and one splice site deletion are analysed. These had previously been associated with canine DCM (Meurs et al., 2012, Philipp et al., 2012, Mausberg et al., 2011). Of these four lie within genes while the remaining three do not. The four which lie within genes are: rs22078677 a SNP on chromosome 10 within the *ARHGAP8* gene, rs22422063 a SNP on chromosome 15 within the *FSTL5* gene, rs22923291 a SNP on chromosome 21 within the *PDE3B* gene, and a splice site deletion within the *PDK4* gene. The SNPs which do not lie within genes are rs21953123 on chromosome 1, rs9216599 on chromosome 5, and rs24025150 on chromosome 37. None of the variants have had their functionality confirmed. Those that are within genes are intronic so do not directly code for proteins, it is possible that they may directly influence the protein, or that they are closely linked to functional variation within the gene (Millar et al., 2010, Stephens et al., 2001). Those not

within genes could be within regulatory elements, or they could be linked to functional variation within genes or regulatory elements (Albert and Kruglyak, 2015, Schaub et al., 2012).

The four genes which contain variants associated with canine DCM are *ARHGAP8*, *FSTL5*, *PDE3B*, and *PDK4*. *ARHGAP8* is a member of the RHOGAP family, members of which have been shown to have functional implications in the organisation of the actin cytoskeleton (Johnstone et al., 2004, Hall, 1998). Mutations within actin itself have been associated with human DCM so it is possible that mutations in genes related to actin function could have implications for DCM development (Olson, 1998). As yet there have been no studies implicating *ARHGAP8* involvement in the actin cytoskeleton, but there have been few studies into *ARHGAP8* at all so it is possible that in time that it will be associated with the actin cytoskeleton. There is currently little evidence of a role of *FSTL5* in cardiac function, but the same gene has been associated with the development of a different heart disease in dogs – mitral valve disease (Stern et al., 2015). However, the role that this gene may have within the heart has not been established. *PDE3B* has been shown to have a role in cardiac function so the SNP has the potential to be a functional variant, or closely linked to a functional variant within the gene, although additional work is required to confirm this (Chung et al., 2015, Palmer and Maurice, 2000). The *PDK4* splice site variant is expected to result in a protein with reduced activity compared to the wild type (Meurs et al., 2012). *PDK4* is involved in fatty acid metabolism which is a key component of normal cardiac metabolism (Stavinoha et al., 2004, Stanley and Chandler, 2002). Due to this it is likely that the splice side mutation has important functional implications for cardiac metabolism.

1.5. Human and Canine Osteosarcoma

1.5.1. Cancer

As outlined earlier, cancer is a leading cause of non-communicable morbidity and mortality throughout the world, second only to cardiovascular disease in the number of deaths of adults between the ages of 30 and 70 years old (Siegel et al., 2015, WHO, 2014). For dogs, the most frequent causes of mortality in order are (i) tumours, (ii) trauma, (iii) locomotor (skeletal, muscles and joints),

(iv) cardiac disease, and (v) neurological disease (Bonnett et al., 2005). While cancer in humans has long been recognised as a disease requiring treatment, cancer in dogs is increasingly becoming a concern to pet owners, with some types of cancer occurring at much higher rates in dogs than people (DeVita and Chu, 2008, Grüntzig et al., 2015, Ranieri et al., 2013, Schneider, 1970). These cases of cancer in dogs themselves require treatment, but can also be effective models for the human disease equivalent (Pinho et al., 2012). Although developing new treatments in companion animals with naturally occurring disease is less contentious than inducing disease in experimental animals, ethical concerns with regards to the treatment of individuals enrolled on studies and gaining informed consent from owners remain (Page et al., 2016).

Cancer is a general term used to describe aberrant cellular growth with the ability to metastasise to other tissues and can affect any organ system or tissue (Hanahan and Weinberg, 2000, Parkin et al., 2010). Cancerous cells have unlimited replicative potential and are able to produce their own growth signals whilst being insensitive to anti-growth signals, they evade apoptosis, have sustained angiogenesis, and invade tissues and metastasise (Cheng et al., 2013, Gonzalez-Villasana et al., 2015, Hanahan and Weinberg, 2000, Moon et al., 2013, Rothwell et al., 2012, Tomlinson et al., 2007, Wong et al., 2015, Zelenay et al., 2015). Disease progression starts with a localised tumour, which if left unchecked can invade tissue locally and metastases to other tissues (Siegel et al., 2015, Yancik, 1993). The growth of a primary tumour may not be lethal, for example there are arguments in favour of merely monitoring low risk prostate cancer as it often does not metastasise and on its own causes limited problems (Dall'Era et al., 2012, Heidenreich et al., 2011). This, however, depends on the location and size of the tumour. Brain tumours for example often lead to seizures and problems with cognitive functioning necessitating treatment wherever possible (Ricard et al., 2012). While primary tumours diagnosed when localised can often be cured by surgery, metastases are inevitably incurable (Minn et al., 2005). It is possible for tumours to metastasise to anywhere in the body, but frequent sites include the liver, bones and lungs (Minn et al., 2005, Mundy, 2002, Rothwell et al., 2012). When tumours metastasise they can cause organ failure, cachexia, and immune suppression

leading to infections, which collectively contribute to death (Bozzetti et al., 2014, Inagaki et al., 1974, Nichols et al., 2012).

Cancer is more common in adults than children, adolescents, and young adults (<30 years old) (Forman et al., 2014, Parkin et al., 2010). Despite this, some types of cancer are more common in younger individuals. In particular bone cancer is most common in the young (less than 20 years old) and elderly (over 60 years old) than individuals of intermediary age (20-60 years old) (Forman et al., 2014, Mirabello et al., 2009a, Parkin et al., 2010). There is a general trend of improving 5 year survival rates when averaged over all types of cancer (Siegel et al., 2013, Siegel et al., 2015). In contrast osteosarcoma, the most common bone cancer, has not shown comparable improvements in mortality rates (Dorfman and Czerniak, 1995, Mirabello et al., 2009b, Siegel et al., 2013). There is thus an urgent need to develop improved treatments and cures for osteosarcoma.

1.5.2. Osteosarcoma

A sarcoma is a tumour originating in tissues derived from the mesoderm, affecting bone, cartilage and connective tissue (Souhami and Tobias, 2005). Osteosarcoma is a sarcoma which produces malignant bone or osteoid tissue, it usually originates in the medullary cavity of the bone, but tumours can also originate on the surface of the bone or be extra-osseous (Klein and Siegal, 2006). There are several different subtypes of osteosarcoma and frequently more than one type is found within a tumour and so classification is based on the predominant type (Klein and Siegal, 2006). The common subtypes are osteoblastic (bony), chondroblastic (cartilaginous), and fibroblastic (resemble atypical fibroblasts), with a range of rarer types including low grade tumours, and tumours that do not originate in the medullary cavity (Klein and Siegal, 2006). The unifying feature of all types of osteosarcoma is that histologically they all produce tumour osteoids (Ragland et al., 2002). Typical osteosarcoma disease progression consists of a primary tumour, usually originating within the medullary cavity, which grows, proliferates, and invades, and left unchecked frequently metastasises to the lungs (Bacci et al., 2008). Prognosis for individuals with metastatic tumours is much poorer than for individuals with only

primary tumours. The 5-year-event-free-survival for individuals with metastatic tumours at diagnosis reported to be 27.4%, while individuals with no metastases at diagnosis the 5-year-event-free-survival is approximately 70% (Bacci et al., 2008, Mirabello et al., 2009b). Human osteosarcoma is rare. There were 217,440 estimated breast cancer cases in the NIH-NCI Surveillance, Epidemiology, Evidence and End Results (SEER) database in 2004 alone, compared to 3482 cases of osteosarcoma in the same database between 1973 and 2004 (Jemal et al., 2004, Mirabello et al., 2009b).

Although osteosarcoma is also not common in dogs, it is much more common in dogs than in people. Between 1995 and 2002 of 394,061 insured Swedish dogs, 764 (0.19%) developed a bone tumour (Egenvall et al., 2007). This is an incidence rate of 27.2 dogs per 100,000 included in the dataset each year. In the comparable Swedish human population, 234 men and 164 women had bone cancer between 1998 and 2002, which is roughly 0.89 cases of bone cancer per 100,000 people each year (Parkin et al., 2010). While the canine data only includes insured dogs it is a large dataset so is likely to be representative of the dog population of the country as whole. The human data records all osteosarcoma cases in the country and the average population size between 1998 and 2002 was calculated from government census data. In addition all other countries in the Cancer Incidence in Five Continents registry report human incidence rates similar to Sweden (Mirabello et al., 2009a, Parkin et al., 2010). Thus the incidence rate of canine bone cancer is 27 times higher than the rate in humans. In both human and canine patients the predominant bone cancer diagnosis is osteosarcoma (Egenvall et al., 2007, Mirabello et al., 2009b). The higher incidence rate of canine osteosarcoma makes the pet dog population an ideal model for human disease. While there is potential for any dog to develop osteosarcoma, a subset of larger dog breeds are at increased risk of developing osteosarcoma (Figure 1.1, (Egenvall et al., 2007)). The prognosis for canine osteosarcoma is poor with one year survival rates following diagnosis and treatment typically less than 45% (Frimberger et al., 2016, Moore et al., 2007, Straw et al., 1991).

In both humans and dogs, effective treatment for osteosarcoma involves surgery to remove primary tumours, combined with neoadjuvant and/or adjuvant radiotherapy and chemotherapy (Selmic et al., 2014, Ta et al., 2009). Surgery can either be amputation or limb salvage surgery (Liptak et al., 2006, Reddy et al., 2015). While limb salvage surgery is most often performed on patients with lower grade tumours the type of surgery does not impact on survival for most human tumours (Reddy et al., 2015). More important prognostic factors are how the tumour responds to chemotherapy and the presence of metastases prior to surgery (Reddy et al., 2015). The advantage of limb salvage surgery over amputation is that those patients treated with limb salvage have better function and quality-of-life than those who have an amputation (Mavrogenis et al., 2012).

1.5.3. Epidemiology and Genetics of Osteosarcoma in Humans

Some cases of human osteosarcoma are associated with heritable cancer syndromes, and the genetic bases of these have been established (Ognjanovic et al., 2012, Stinco et al., 2008, Thomas and Ballinger, 2015). Most instances of osteosarcoma, however are not associated with heritable cancer syndromes and there have only been two heritable genetic loci identified, but there are some risk factors associated with osteosarcoma development including growth, puberty, sex, and race (Mirabello et al., 2009b, Mirabello et al., 2011, Savage et al., 2013). Evidence that growth plays a part in the development of osteosarcoma comes from the age of onset frequently coinciding with rapid bone growth during puberty, tumour sites most frequently at the end of bones where active growth occurs, and people affected by osteosarcoma are on average taller than the unaffected population (Mirabello et al., 2009b, Mirabello et al., 2011). There is a difference between sexes in the development of osteosarcoma with males more commonly affected than females, and there is some evidence of variation in the incidence rate between races in the USA (Mirabello et al., 2009b, Mirabello et al., 2011). Although osteosarcoma itself does not appear to be directly heritable in most instances, there are heritable components to the risk factors (Yang et al., 2010).

There have been somatic mutations in tumour suppressor genes identified in individuals with heritable cancer syndromes, and there have been mutations identified in osteosarcoma tumours compared to non-tumours (Chen et al., 2014, Ognjanovic et al., 2012, Srivastava et al., 1990, Zhang et al., 2015). There have, however, only been two somatic genetic mutations associated with osteosarcoma specifically (Savage et al., 2013). This lack of identified somatic genetic associations is not surprising based on the lack of heritability observed in human osteosarcoma. Despite the lack of heritability and somatic genetic mutations identified there have been over 900 genes associated with human osteosarcoma (Poos et al., 2014). These genes have been associated with osteosarcoma due to either differences in expression between tumour and non-tumour tissue, or due to mutations that have arisen in the tumour tissue compared to the non-tumour tissue (Man et al., 2004, Xiao et al., 2015, Yuan et al., 2013). Mutations in osteosarcoma tumour tissue, but not non-tumour tissue, and differences in gene expression between tumour and non-tumour tissue could be either the cause of the tumour or as a result of the tumour. A possible cause of both differences in expression between tumour and non-tumour tissue, and mutations in tumour but not non-tumour tissue, is genomic and chromosomal instability (de Bruin et al., 2014, Galanos et al., 2016). Genomic and chromosomal instability is a reported factor in many types of cancer progression (de Bruin et al., 2014, Galanos et al., 2016). Osteosarcoma has been shown to display chromosomal instability associated with mutations in the *TP53* gene (Al-Romaih et al., 2003). A consequence of this chromosomal instability is aneuploidy, which can lead to the overexpression of some genes within malignant cells, disrupting normal cell processes (Carter et al., 2006). Although mutations in *TP53* appear to be associated with chromosomal instability, the gene itself does not seem to be subsequently over expressed following aneuploidy (Al-Romaih et al., 2003, Carter et al., 2006).

1.5.4. Epidemiology and Genetics of Osteosarcoma in dogs

As outlined above, canine osteosarcoma is more common than human osteosarcoma (Egenvall et al., 2007, Mirabello et al., 2009a, Parkin et al., 2010). Despite this there are similar risk factors associated with the development of osteosarcoma in dogs as in people, including growth and sex

(Egenvall et al., 2007, Ru et al., 1998). In addition, osteosarcoma in dogs is also influenced by breed and neutering status (Egenvall et al., 2007, Ru et al., 1998). Large dogs make up the majority of osteosarcoma cases, reflecting the human population where individuals with osteosarcoma are more likely to be taller than average (Egenvall et al., 2007, McNeill et al., 2007, Mirabello et al., 2011). In the canine population, as with the human population, there also appears to be a skewed sex ratio with males more likely to develop osteosarcoma than females (Egenvall et al., 2007, Mirabello et al., 2009a). Neutering status, although not relevant in the human context, appears to contribute to osteosarcoma risk where neutered dogs are more likely to develop osteosarcoma than non-neutered dogs (Ru et al., 1998). This, combined with the association with puberty, suggests a complex role for sex hormone signalling in osteosarcoma risk. In contrast to human osteosarcoma, canine osteosarcoma appears to be heritable, with some breeds appearing to be predisposed to developing osteosarcoma compared to others (Egenvall et al., 2007, Phillips et al., 2007). Interestingly of the 15 breeds with the highest reported incidence of osteosarcoma 12 are within a clear clade on the canine phylogeny (Egenvall et al., 2007, vonHoldt et al., 2010). This relationship between affected breeds could indicate a potential common genetic origin of canine osteosarcoma across breeds, however the clade is large and contains many breeds that are not highly affected by osteosarcoma, thus this seems less likely (vonHoldt et al., 2010).

There have been 34 genetic loci associated with canine osteosarcoma across four breeds (Karlsson et al., 2013, Phillips et al., 2010). Karlsson *et al.* (2013) identified 33 loci as associated with canine osteosarcoma across three breeds and Phillips *et al.* (2010) identified a single locus associated with osteosarcoma in Deerhounds. None of the loci were consistently associated across breeds, further suggesting that there may be a difference between breeds in the genetic predisposition to developing canine osteosarcoma (Karlsson et al., 2013, Phillips et al., 2010). Currently none of the genetic variants identified as associated with canine osteosarcoma have had their mechanism of action verified. Verification of the mode of action could lead to novel therapeutic targets, and if these loci can be shown to consistently predict increase risk of

osteosarcoma the number of affected individuals could be reduced. Breeders could genotype prospective sires and dams prior to mating and choose parents to reduce the risk of offspring having multiple deleterious osteosarcoma alleles. In addition to the 34 genetic loci identified as associated with canine osteosarcoma, there have been genes identified as differentially expressed in canine osteosarcoma compared to non-tumour tissue that have implications for growth and metastasis, and are potential drug targets (McCleese et al., 2013, Millanta et al., 2012, Pang et al., 2014, Shahi et al., 2015). These genes have been identified utilising canine osteosarcoma tumour tissue, and canine osteosarcoma cell lines. There has also been variation in the expression of genes within tumours associated with survival time in canine osteosarcoma (Maniscalco et al., 2015, Selvarajah et al., 2009, Selvarajah et al., 2012, Selvarajah et al., 2013).

Additional work is required to confirm the effect of the genetic loci identified as associated with canine osteosarcoma, and account for the additional variation observed in the development of disease (Karlsson et al., 2013, Phillips et al., 2010). There are several breeds that appear to have a genetic predisposition to osteosarcoma for which no genetic loci have been identified which could benefit from the identification of osteosarcoma predisposition loci (Egenvall et al., 2007). In addition further work is required to establish improved treatment regimens for individuals that develop disease, the identification of particular genetic pathways that are altered in osteosarcoma tumour tissue compared to non-tumour tissue could facilitate this. Any genetic loci identified as associated with canine osteosarcoma have the potential to be examined for an association with human osteosarcoma, and any treatments shown to be effective in either species could be applied to the other.

1.5.4.1. Discussion of selected breeds affected by osteosarcoma

1.5.4.1.1. Rottweiler

The Rottweiler is a large breed of dog with a high incidence of osteosarcoma (Egenvall et al., 2007). The breed originated in Germany from mastiff type dogs used for droving and draught and is part of the UK Kennel Club's Working Breed

Group (The Kennel Club UK, 2016a). Following the creation of breed standards in the 1930s when the breed was registered with the UK and American Kennel Clubs it is likely that Rottweilers have exclusively been mated with other Rottweilers leading to a closed gene pool within the pedigree Rottweiler population (American Kennel Club, 2016, The Kennel Club UK, 2016a). Within the insured Swedish dog population Rottweilers have the 5th highest incidence ranking for osteosarcoma with a median age of onset of 7.9 years (Egenvall et al., 2007). There have been 15 loci associated with the development of osteosarcoma in Rottweilers (Karlsson et al., 2013).

1.5.4.1.2. Irish Wolfhound

The Irish Wolfhound is the breed with the highest prevalence of osteosarcoma within the insured Swedish dog population (Egenvall et al., 2007). In addition it also has one of the lower median ages of onset at 6.6 years, only the greyhound has a lower median age of onset at 6.2 years (Egenvall et al., 2007). The Irish Wolfhound is a giant breed of dog whose history includes a period when they were close to extinction (Samaha, 1991). In the process of conserving the breed the few remaining Irish Wolfhounds were crossed with Great Danes, Scottish Deerhounds, Borzois, and Mastiffs (Philipp et al., 2012, Samaha, 1991). This out-crossing will have introduced some genetic diversity, but to retain the Irish Wolfhound phenotype a large amount of inbreeding will have been required, therefore as with most modern breeds genetic diversity is low within the breed (Jansson and Laikre, 2014). Importantly for the Irish Wolfhound breed both the Great Dane and Deerhound breeds have a high prevalence of osteosarcoma, and these were two breeds used to help rescue Irish Wolfhounds from extinction (Egenvall et al., 2007, Phillips et al., 2007, Samaha, 1991). Combined with inbreeding, the use of two breeds prone to the development of osteosarcoma could have led to Irish Wolfhounds acquiring osteosarcoma predisposition genetic variants from both breeds, increasing the risk of disease within Irish Wolfhounds. There have been 4 loci associated with the development of osteosarcoma in Irish Wolfhounds (Karlsson et al., 2013).

1.6. Overall aims and hypotheses

In this thesis multiple hypotheses relating to the molecular characterisation of canine DCM and osteosarcoma were tested. These diseases have not previously been reported to have a common underlying mechanism. However, their high prevalence in mutual breeds leads to the hypothesis that a shared genetic and mechanistic process influences the development of both diseases in dogs.

One aim of this study was to improve the genetic knowledge to support the future development of genetic tests to identify susceptible dogs prior to the development of disease. The identification of susceptible individuals could lead to increased health monitoring and early intervention strategies, but also modified breeding strategies to reduce the risk of offspring developing disease. New treatments targeting affected genetic pathways could also be developed if causative mutations and/or differentially expressed genes are identified. Secondly dogs represent ideal models of human disease. Thus any genetic loci or treatments identified in the canine population could be relevant to disease in people.

1.6.1. Hypothesis 1. Multiple loci interact in DCM risk in dogs

There have been multiple loci in >50 genes associated with the development of human DCM (Posafalvi et al., 2012). Despite clear heritability and breed predispositions there have only been 10 loci associated with the development of canine DCM (Egenvall et al., 2006, Mausberg et al., 2011, Meurs et al., 2012, Meurs et al., 2013, Philipp et al., 2012, Schatzberg et al., 1999). As discussed earlier, this could be attributable to inappropriate sample sizes, inappropriate unaffected control individuals, or the development of disease being dependent on genotypes at multiple interacting loci (Simpson et al., 2015b). In this thesis appropriate cases and control individuals will be identified and the hypothesis that multiple loci act together to influence disease progression will be tested. Validation of this hypothesis could impact on genetic testing prior to breeding in animals, but could also be of use in human genetic counselling.

1.6.2. Hypothesis 2: The transcriptome of non-malignant and osteosarcoma specimens will differ thereby identifying potential novel biomarkers of disease

Osteosarcoma is a rare disease that does not appear to have modifiable risk factors in people which limits the development of effective prevention strategies (Mirabello et al., 2009b, Mirabello et al., 2011). Thus identifying early diagnostic and improved treatment approaches are the key focus to improving outcomes in people. While there are some genetic loci associated with canine osteosarcoma (Karlsson et al., 2013) these are limited and research into improved diagnosis and treatment options is also warranted in the canine population. A potential method for identifying new treatments is by determining genes that are aberrantly expressed in tumour tissue compared to normal tissue which could lead to the development of drugs targeting particular pathways (Calon et al., 2015, Pfefferle et al., 2016, The Cancer Genome Atlas Research Network, 2014). The hypothesis that there is differential gene expression in canine osteosarcoma tumour tissue and non-tumour tissue was tested in this thesis. Genes identified as aberrantly expressed could represent novel disease biomarkers or therapeutic targets in canine and human osteosarcoma.

1.6.3. Hypothesis 3: Polymorphic polyglutamine repeats are associated with DCM and osteosarcoma

As outlined earlier, breeds with increased risk of osteosarcoma are also breeds that have increased risk of DCM (Figure 1.1) (Egenvall et al., 2006, Egenvall et al., 2007). Given the sex disparity of both osteosarcoma and DCM, this led to the hypothesis that a single underlying endocrine mechanism related to androgen signalling increases the risk of both diseases in the highly affected breeds. To test this hypothesis the association of the polyglutamine repeat tracts of the androgen receptor (*NR3C4*) and *NCOA3* genes with both DCM and osteosarcoma in Irish Wolfhounds and Rottweilers was examined.

1.6.4. Hypothesis 4: Fatty acid profiles differ between cardiac diagnoses

Differences in genetic predisposition and expression ultimately lead to different phenotypes. These phenotypes can include developing DCM, osteosarcoma,

or other diseases, or remaining healthy into old age. Many diseases, including heart disease, have an inflammatory component to them, of which fatty acid derived eicosanoids contribute towards (Bagga et al., 2003, Danesh et al., 2000, Harris, 2009, Mohler et al., 2001). The hypothesis that individuals with different diseases have different blood fatty acid profiles was tested. This was done by establishing fatty acid profiles from DCM affected dogs, dogs with other cardiac diseases, and dogs with additional non-cardiac diagnoses.

Chapter 2 Materials and Methods

2.1. Ethics

This study was approved by the University of Nottingham ethics committee in compliance with Home Office regulations and the Veterinary Surgeons Act. Ethics Numbers: University of Nottingham 1823 160714 and 959 130925. Informed consent was obtained from all dog owners involved in this research. A copy of the approved consent form is in Appendix 2. Completed consent forms are retained securely in the School of Veterinary Medicine and Sciences.

2.2. Sample acquisition and storage

2.2.1. DNA Swabs

Buccal swabs were obtained from 564 Irish Wolfhounds (IWH) and 1019 Rottweilers. Buccal swabs were taken using Isohelix DNA Buccal Swabs (Cell Projects Ltd. UK). These swabs were taken by members of the research group, owners, and vets. The swab was inserted into the dog's mouth and rubbed on the cheek for up to 2 minutes to enable optimum cell collection. Swabs were stored as directed by the manufacturers, this was at room temperature for Rottweiler samples and room temperature upon collection followed by 4°C for IWH samples. Any swabs stored for longer than a month prior to DNA extraction were stored at -20°C. Optimisation trials carried our prior to collection of these swabs indicated that appropriate quality and quantity of DNA could be obtained following all storage methods.

2.1.1. Osteosarcoma tumours

Osteosarcoma tumour samples were collected by collaborating vets at the practices where patients were being treated. These were either collected as part of treatment during amputation or immediately following euthanasia. Amputation or euthanasia was carried out purely as normal veterinary treatment and not as an impact of inclusion in the study. A $1cm^3$ piece of tumour was extracted along with a $1cm^3$ piece of adjacent non-tumour tissue. These samples were placed directly into RNA*later*® (Sigma-Aldrich, USA) and stored at room temperature during shipping to the laboratory. Upon arrival the samples

were stored at -20°C. Osteosarcoma tumour and non-tumour tissue was obtained from a total of 8 individuals, five Rottweilers and three Deerhounds.

2.1.2. Sample curation

Each sample was given a unique identification number. During sample collection, associated information about the individual was also collected. A copy of the sample information form is shown in Appendix 3. This information was stored in a database associated with the sample number. The unique number enabled blinded experimentation and anonymization, whilst ensuring scientific accuracy.

Follow-up data on the health of DNA-sampled dogs was obtained at numerous time points. Owners were asked to fill in a 'health update' form annually or following any change in health/upon death. This was provided as both online and paper formats to ensure maximum owner participation, a copy of this form is in Appendix 3. In addition, telephone surveys were carried out in collaboration with undergraduate research project students who were supervised by the PIs on this canine health project. The surveys were utilised to obtain health updates and epidemiology data on all dogs from which samples had been obtained. A copy of the questionnaire is in Appendix 4. Many IWHs in the UK are presented to specialist veterinary cardiologists on a regular basis for heart disease screening as part of a breed society subsidised programme. These heart screenings consist of cardiac auscultation, a six lead electrocardiogram, and complete echocardiographic assessment. Data from these heart screenings were provided by veterinary cardiology specialist Dr Serena Brownlie. Cardiac diagnoses were determined by Professor Malcolm Cobb based on the data from heart screenings. Information from owner health updates and heart screenings was added to that stored in the database. Owners were kept up to date with research progress, encouraged to enter the study, and informed/reminded about health update forms via numerous outreach projects. These included newsletter articles and website updates (via Rottweiler and IWH health and breed groups), at owner events led by our group at University of Nottingham, at heart testing days, via social media, and at events organised by breed groups which we attended.

2.2. DNA extraction from buccal swabs

In order to extract the DNA from buccal swabs, the end of the swab was first dissociated into small pieces using scissors which were sterilised between swabs using Anistel (Tristel, UK) and then wiped down with Industrial Methylated Spirits (IMS) (Thermo Fisher Scientific, USA). The small pieces of swab were put into a sterile 1.5ml microcentrifuge tube (Eppendorf, Germany). The DNA-containing swab fragments were re-suspended in 600µL of a solution composed of 5% sodium Chelex® 50-100 mesh (dry) (Sigma-Aldrich, USA) reconstituted in 1xTE (10mM Tris (Thermo Fisher Scientific, USA)), 1mM EDTA (Thermo Fisher Scientific, USA) in H_2O, pH 8.0) and 20µL of 10mg/mL proteinase K (Promega, USA), 50mM Tris (Thermo Fisher Scientific, USA), 10mM $CaCl_2$ (Thermo Fisher Scientific, USA) pH 8.0. The sample-containing microcentrifuge tube was vortexed to mix the components. The microcentrifuge tube was then placed in a water bath at 45°C overnight to digest the cells. The sample was then vortexed once more before incubation in a heating block at 100°C for 8 minutes to inactivate the proteinase K. The sample was then centrifuged at 13,500xg for 3 minutes to separate the Chelex® and swab from the supernatant. The DNA-containing supernatant was then transferred into a sterile 1.5mL microcentrifuge tube and was used in subsequent PCRs. The extracted DNA was then stored at -20°C in the Nottingham Comparative Genomic Biobank.

2.3. RNA extraction, quantification, sequencing, and qRT-PCR

2.3.1. RNA extraction

RNA was extracted from osteosarcoma tumour and associated non-tumour tissue samples using a Qiagen RNeasy Mini Kit (Qiagen, Germany). For each RNA extraction approximately 350mg of tissue was cut into small pieces using a sterile scalpel before placing in a GentleMACS tube (Miltenyi Biotec, Germany) with 1mL of TRIzol® (Thermo Fisher Scientific, USA). The tissue was homogenised using a GentleMACS dissociator (Miltenyi Biotec, Germany) at 1,000rpm for 1 minute and the homogenate was then transferred to a 1.5mL microcentrifuge tube (Eppendorf, Germany). The homogenate was incubated at room temperature (15-25°C) for 5 minutes before adding 200µL chloroform (Sigma-Aldrich, USA). The homogenate/chloroform mixture was shaken

vigorously for 15 seconds to fully mix, before incubating at room temperature for 2-3 minutes and then centrifuging at 12,000x g for 15 minutes at 4°C. The upper aqueous phase was transferred to a new 1.5mL microcentrifuge tube and 1 volume of 70% ethanol was added followed by thorough mixing by vortexing (Topmix FB15012, Thermo Fisher Scientific, USA).

The RNA sample (600µL) was then transferred to the RNeasy Mini spin column placed in a 2mL collection tube which was centrifuged at 8,000x g for 15 seconds and the flow through was discarded. The remaining sample was transferred to the RNeasy Mini spin column and centrifuged at 8,000x g for 15 seconds and the flow through discarded. On column DNase digestion was performed. Firstly 350µL of Buffer RW1 was added to the RNeasy Mini spin column and centrifuged at 8,000x g for 15 seconds in order to wash the membrane. 10µL of DNase I was added to 70µl of Buffer RDD in a clean 1.5mL mircocentrifuge tube, this was added directly to the RNeasy Mini spin column membrane and incubated at room temperature for 15 minutes. 350µL of Buffer RW1 was then added to the RNeasy Mini spin column and then centrifuged at 8,000x g for 15 seconds. Buffer RPE (500µL) was added to the RNeasy Mini spin column and centrifuged at 8,000x g for 15 seconds to wash the membrane, the flow through was discarded. This 500µL Buffer RPE step was repeated and the flow through and collection tube were discarded. The RNeasy Mini spin column was then placed in a new collection tube and centrifuged at 17,000x g for 1 minute, the flow through and collection tube were discarded. The RNeasy Mini spin column was then placed in a clean 1.5mL microcentrifuge tube and 33µL of RNase free H_2O was added directly to the membrane. To elute the RNA from the membrane this was centrifuged at 8,000x g for 1 minute. To maximise yield the elutate was then added to the membrane and centrifuged at 8,000x g again. The extracted RNA was stored at -80°C.

2.3.2. RNA quantification

RNA was initially quantified using a NanoDrop 8000 (Thermo Fisher Scientific, USA), before more accurate quantification and quality assurance using an Agilent 2100 Bioanalyzer (Agilent, USA). The Agilent 2100 Bioanalyzer is only accurate for RNA concentrations between 25 and 500ng/µl so based on the

NanoDrop 8000 results the RNA was diluted appropriately prior to Agilent 2100 Bioanalyzer analysis.

2.3.3. RNAseq

RNAseq was completed at Edinburgh Genomics using an Illumina HiSeq platform. In all cases >20 million reads were obtained from all samples analysed. The resultant raw fastq reads were processed for quality (phred score >30) and adapter sequences removed using Trimgalore. The processed raw reads were aligned to the canine genome (CanFam3.1) using the Ensembl annotated iGenomes build using Tophat2 (Kim et al., 2013a). Differential gene expression analysis was completed using Cufflinks/Cuffdiff (Trapnell et al., 2013). The RNAseq differential gene expression analysis was conducted by Dr. N. Mongan. Genes were selected for qPCR validation based on implication in cancer, biological function, statistical significance ($p<0.05$), and a fold change >2.

2.3.4. Quantitative Reverse Transcriptase PCR

cDNA was generated from the RNA extracted from osteosarcoma tumours and associated non-tumour tissue by reverse transcriptase PCR. The reverse transcriptase PCR reaction mixture consisted of 2µL of 5x qScript™ cDNA SuperMix (Quanta Biosciences, USA), 0.5-1µg RNA, adjusted to 10µL using PCR grade H2O. Reverse transcriptase PCR was carried out in a water bath (Sub Aqua Pro, Grant Instruments, UK) at 25°C for 5 minutes followed by 42°C for 1 hour.

Gene expression was quantified using qRT-PCR. The reaction mixture consisted of 0.65µL TaqMan hydrolysis probe and primers, 7.5µL 2x Roche Probe Master Mix (Roche, Switzerland), 4.85µl H_2O, 2µl cDNA. Each sample was run in triplicate on a LightCycler 480 (Roche, Switzerland) PCR machine. Quantification of expression was performed using LightCycler 480 software (Roche, Switzerland). Expression for all samples were normalised to the internal control Actin (Actin qRT-PCR performed by Dr. N. Mongan) and relative expression of tumour compared to non-tumour tissue was calculated utilising the methods described by Pfaffl (2001). *t*-tests were performed using GraphPad

Prism 7.01 for Windows (GraphPad Software, La Jolla California, USA) to establish whether there were significant differences in gene expression between the tumour and non-tumour tissue.

Gene ontology, KEGG pathway, Wiki Pathway, and transcription factor target analyses were performed by WebGestalt software to establish whether there were differentially expressed genes with common functions (Wang et al., 2013a, Zhang et al., 2005). These analyses were carried out utilising all genes identified as statistically significantly different ($p<0.05$) between tumour and non-tumour tissue, and with a fold change >2.

2.4. IWH DCM and AF relationship

Individuals diagnosed with DCM, AF, and both DCM and AF combined in the database were identified, and the numbers in each diagnosis category "DCM", "AF", and "DCM and AF" were determined. In the subset of individuals with both DCM and AF, the approximate time from AF diagnosis to DCM diagnosis was established based on the dates of the heart screenings at which each were diagnosed. Individuals that were diagnosed with AF, but not DCM had the approximate time with AF determined by the time between the first heart screening that AF was diagnosed at and the most recent heart screening. A *t*-test was performed using R statistical software (RCoreTeam, 2015) to establish whether the time from AF diagnosis to DCM diagnosis was significantly different from the time that individuals had a diagnosis of AF, but not DCM

2.5. Sex differences in numbers affected and age of disease onset

The number of IWH males and females diagnosed with DCM or AF was established from the database along with the number of males and females not diagnosed with DCM or AF. A Chi-Square (χ^2) test was performed to establish whether there were differences in the number of males and females diagnosed with DCM or AF. The age at first diagnosis of DCM or AF was established for all individuals diagnosed with either disorder. A *t*-test was performed using R statistical software (RCoreTeam, 2015) to identify statistical differences between males and females at age of diagnosis. The data was also displayed

visually in bar plots generated using R statistical software (RCoreTeam, 2015). The age by which 80% of all affected individuals had been diagnosed with DCM or AF was established for each sex separately. This analysis was repeated for osteosarcoma age of onset in IWH and Rottweilers separately, with a 90% age restriction for Rottweilers due to greater numbers of Rottweilers getting to old age compared to IWHs.

2.6. IWH DCM associated SNP genotyping

2.6.1. SNP primer design

Primers were designed for five SNPs that had previously been associated or putatively associated with IWH DCM in order to amplify the region containing the SNP and allow specific restriction enzyme digestion for one of the alleles of interest (Philipp et al., 2012). The locations of each of the five SNPs reported by Philipp *et al.* (2012) were based on the canine genome build version 2. By entering the SNP location into the UCSC Genome Browser (Kent et al., 2002) the dbSNP identifier was obtained and from dbSNP the surrounding sequence obtained. The region containing the SNP was examined for a restriction enzyme site specific to one of the alleles at the SNP using NEBcutter (Vincze et al., 2003). If there was a restriction site, PCR primers were designed to amplify a region of 300-400 base pairs with the SNP roughly in the centre of the fragment. If there was no restriction site, a restriction site was designed into one of the primers by changing one or two of the bases in the primer sequence. Certain rules were followed when designing PCR primers these were: length of roughly 24 nucleotides long, ideally ending in a C or G base, ideally roughly 50%G/C content, melting temperature of 72°C (+/-4°C), ideally the same melting temperature for both primers in the pair, avoiding long chains of a single nucleotide and repetitive elements, not complimentary base at the start and end of a primer. Some samples did not work for the original primers so additional primers were designed to enable successful PCR of the majority of samples, if the restriction site was designed into the primer sequence only a single new primer was designed. Primers were obtained from Sigma-Aldrich (UK). Primer information and fragment size amplified is presented in Table 2.1.

Table 2.1. PCR primer information.

SNP	Primer pair	Forward primer sequence	Reverse primer sequence	PCR fragment length
Chr1: rs21953123 C/T	initial primers	TCCTCTCCAAACATTAGAAAAAGACC	ACTACATATAGTCAGCTTTACAGATTG	311bp
	additional primers	TATCGTACTTTATAATGAAAGATTGAATG	AGAATTTAATATCTGAAAATATATCAACAC	417bp
Chr10: rs22078677 A/C	initial primers	CCTGGTGTCAGAAACACAAGgCAC	TCAAGTAGCTTAAACTCTCAGGGCC	374bp
	initial forward primer, additional reverse primer		TGTGTATTCATCAGCTCTTCCTAGG	161bp
Chr15: rs22422063 C/A	initial primers	AATCATTTGACAGTTAGGTTAACTTCC	CACAATTTGCACATTACACATTATTAAC	375bp
	initial forward primer, additional reverse primer	GCTGGTAAACATTCTTAAATTCAGCG	ATTTGAGTTTTCCCTCACATTATTGAC	443bp
Chr21: rs22923291 G/A	initial primers	GAAACTCCAGTCATGTTTAACTATTTG	GAAGAGCAGTAATGACTAATACgCAG	349bp
	additional forward primer, initial reverse primer	TGTTCATCTCTCACAGGACTTGAG		410bp
Chr37: rs24025150 G/A	initial primers	GGAAACTGGTTTCCAATGAAGAcC	ACGTAACAAGTTTGAAGTGTTGCTG	374bp
	initial forward primer, additional reverse primer		CCTGCCACCTGTGTTACTTTACGC	133bp

DCM associated dbSNP identifier (from Philipp *et al.* (2012)), PCR primer sequence information, if the primer pair is the initial or new primers, and resulting PCR product sizes. Primer sequence in lower case indicates a base change from the reference sequence to create a restriction digest site.

2.6.2. DCM SNP PCR

The PCR reaction mixture consisted of the following: 1x LightCycler® 480 Probes Master Mix (Roche, Switzerland), primers at a concentration of 0.5µM, between 2 and 4µL of template DNA per reaction, adjusted to 25µL using PCR grade H_2O. The standard volumes for a 25µL reaction was 12.5µL 2x Roche Probe Master Mix, 5.5µL H_2O, 2.5µL of each primer from a stock with concentration of 5µM and 2µL template DNA. Where PCR reactions initially failed, increased template DNA was used (up to 4µL) and the amount of H_2O in the reaction was reduced accordingly. PCR reactions were carried out in a Techne 512 or 412 thermo cycler (Bibby Scientific Limited, UK) under the following conditions: Initial denaturation 94°C for 30 seconds, 40 cycles of: 94°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 30 seconds, final extension 72°C for 5 minutes. The annealing temperature optimisation was determined by performing a temperature gradient PCR between 52°C and 64°C. Annealing temperatures for each SNP are in Table 2.2. 5µL of PCR products were visualised on 1.5% agarose 1X TAE gels stained with Nancy-520 (Sigma-Aldrich, UK) run at 120v for 25 minutes in an OWL EasyCast gel electrophoresis tank (Thermo Fisher Scientific, USA) in 1X TAE buffer. PhiX174 DNA-Hae III digest ladder (NEB, USA) was used for sizing. At least one sample for each primer pair was Sanger sequenced by Source Bioscience (Nottingham, UK) and confirmed to be the target sequence by alignment with the reference sequence obtained during primer design using BioEdit (Hall, 1999) and BLAST (Altschul et al., 1990) prior to commencing analysis of large numbers of samples.

Table 2.2. PCR annealing temperatures as determined by temperature gradient PCR optimisation for each primer pair.

SNP	Primer pair	Annealing temperature
Chr1: rs21953123 C/T	all primer pairs	52°C
Chr10: rs22078677 A/C	initial primers	60°C
	initial forward primer, new reverse primer	52°C
Chr15: rs22422063 C/A	all primer pairs	52°C
Chr21: rs22923291 G/A	initial primers	58°C
	new forward primer, initial reverse primer	52°C
Chr37: rs24025150 G/A	initial primers	58°C
	initial forward primer, new reverse primer	52°C

2.6.3. Restriction digests and genotyping

The reaction mixture for each restriction digest consisted of 3µL of 10x Buffer, 20µL PCR product, and 1-6 units of enzyme in a reaction volume of 30µL. The enzyme manufacturer provided the appropriate buffer for each enzyme and the stated activation temperature, the number of units of enzyme was optimised for each restriction digest reaction. The details for each restriction digest are shown in Table 2.3. The restriction digests were performed by incubating the reaction mixture at the activation temperature of enzyme for 14 hours.

Following digestion, the products were run on 2% agarose 1X TAE gels stained with Nancy-520 (Sigma-Aldrich, UK) run at 100v for 45 minutes in an OWL EasyCast gel electrophoresis tank (Thermo Fisher Scientific, USA) in 1X TAE buffer. A DNA size ladder (ϕx-HaeIII fragments) purchased from NEB was used to determine the size of the digested PCR fragments. Genotypes were determined by the restriction digestion fragment pattern based on the presence of an appropriate restriction site determined by the presence of the SNP, with the possibility of detecting homozygotes (AA, aa) and heterozygotes (Aa). Presumptive homozygotes for each genotype (AA, aa) were confirmed by direct Sanger sequencing of representative PCR fragments (Source BioSciences, Nottingham). For all loci a single band the same size as the PCR product showed that none of the PCR product had been digested and was genotyped as a homozygote for the allele that does not digest (Figure 2.1A). The absence of this larger band and the presence of smaller bands at the expected sizes following digestion (shown in Table 2.1) showed that all of the PCR product had been digested so these were genotyped as homozygotes for the allele that was digested by the restriction enzyme (Figure 2.1C). Those with a band the same size as the PCR product plus bands at the expected sizes following successful digestion in Table 2.1 were genotyped as heterozygotes (Figure 2.1B). These genotypes were manually recorded within a database alongside appropriate sample numbers and clinical information. One sample of each genotype at each SNP was confirmed by Sanger sequencing by Source Bioscience (Figure 2.2) (Nottingham, UK).

Table 2.3. Restriction digest information.

SNP	Enzyme	units of enzyme per reaction	Restriction site	cuts allele	Primer pair	Digest fragment sizes
Chr1: rs21953123 C/T	HpyCH4III (NEB, USA)	3	ACTGT	C	original primers	228 & 83
					new primers	260 & 157
Chr10: rs22078677 A/C	BanI (NEB, USA)	6	GgCACC	C	original primers	19 & 355
					original forward primer, new reverse primer	19 & 142
Chr15: rs22422063 C/A	NciI (NEB, USA)	6	CCCGG	C	original primers	196 & 179
					original forward primer, new reverse primer	227 & 216
Chr21: rs22923291 G/A	Fnu4HI (NEB, USA)	1	GCTGc	G	original primers	310 & 39
					new forward primer, original reverse primer	384 & 39
Chr37: rs24025150 G/A	HpaII (NEB, USA)	3	cCGG	G	original primers	28 & 346
					original forward primer, new reverse primer	28 & 105

Enzymes, restriction sites, primer pairs and digest fragment sizes for each SNP.

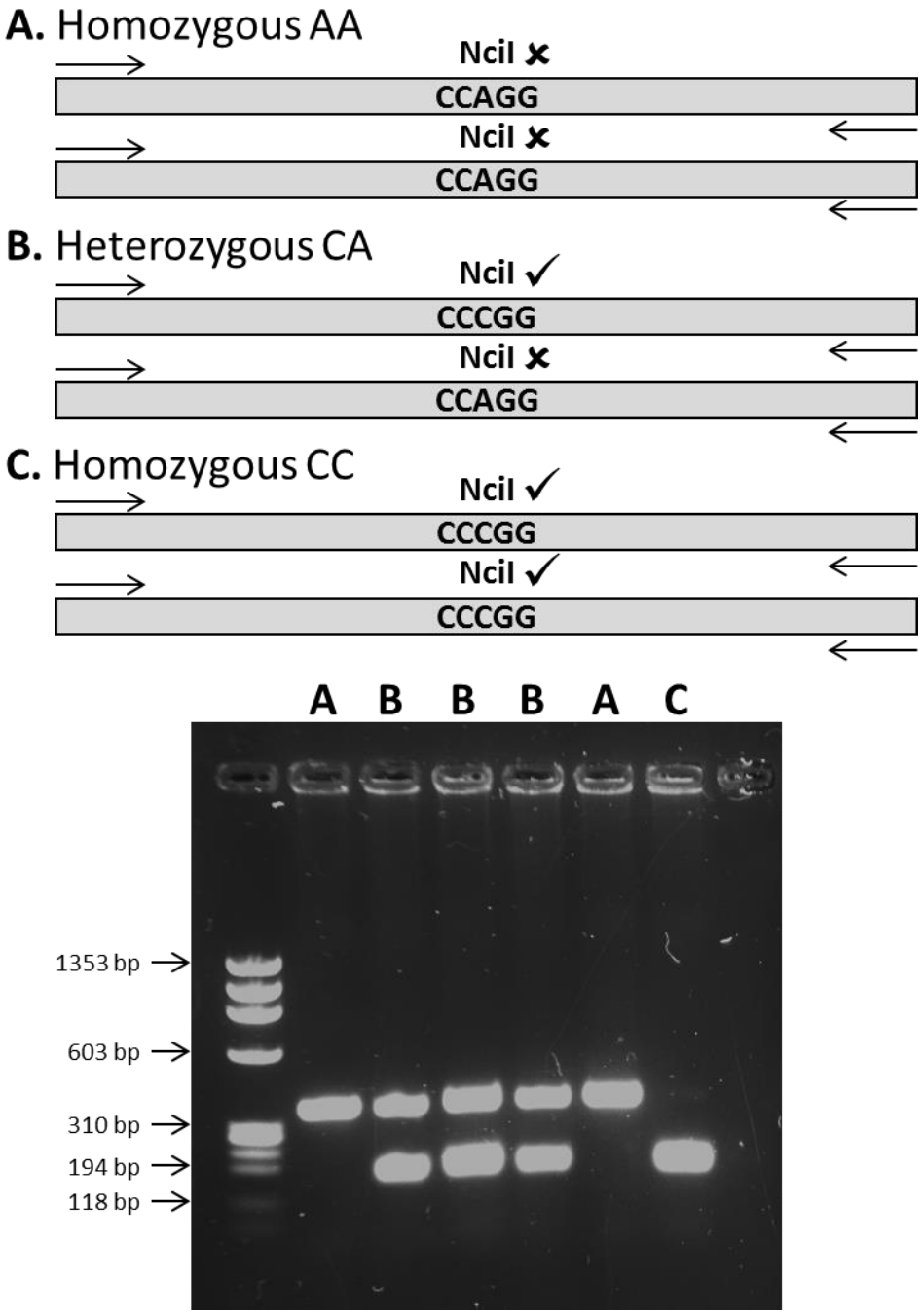


Figure 2.1. Example of how SNPs were genotyped by restriction digest. A. Neither copy contains the restriction site so neither are digested by the restriction enzyme resulting in a single DNA fragment identical in size to the PCR product. B. One copy contains the restriction site so is digested by the restriction enzyme to smaller fragments, the other copy does not contain the restriction site so is not digested by the restriction enzyme, this results in one band the same size as the PCR fragment and smaller bands* equivalent to the digest fragment sizes. C. Both copies contain the restriction site so both are digested by the restriction enzyme to smaller fragments*.
*In this example the two fragments that result from the restriction digest are similar in size so are not separated on the electrophoresis gel.

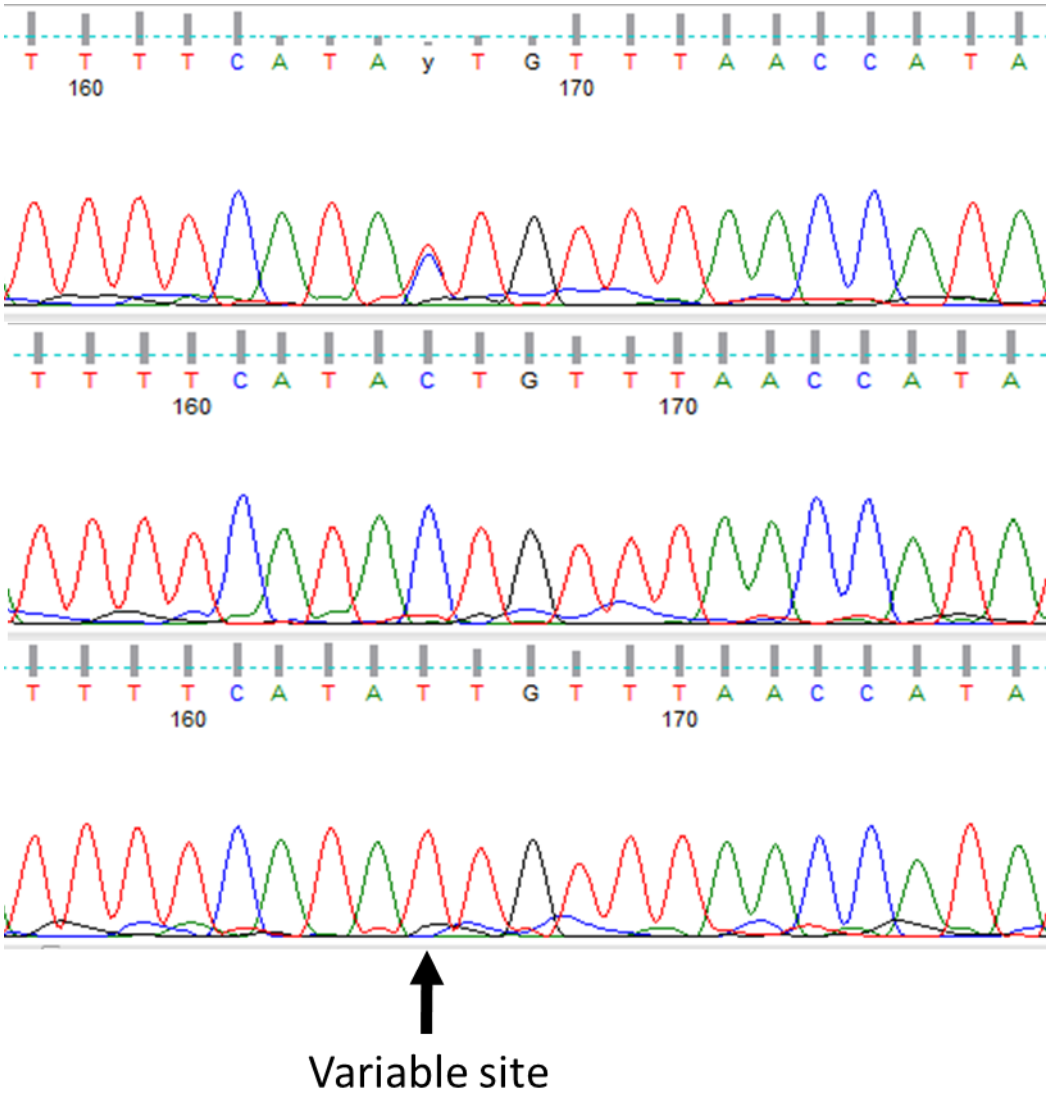


Figure 2.2. Example of SNP sequencing chromatograms. This is the SNP on chromosome 1 rs21953123 C/T. The variable site in the sequence is indicated by the arrow. The top individual is heterozygous, the middle is homozygous C, and the bottom individual is homozygous T.

2.7. Testing SNPs for associations with disease

To establish whether the population within this study genetically resembled the published data by Philipp *et al.* (2012), the allele frequencies at each locus were determined in the entire sampled population irrespective of age or disease status. These allele frequencies were compared to the published allele frequencies at the same loci.

AF was established to be a precursor of DCM (Chapter 4), thus all individuals with a diagnosis of DCM or AF were included in the affected group. From the age of onset analysis (Chapter 4) males were shown to be affected by DCM and AF at an age earlier than females. For this reason, females were considered to be unaffected if they had not been diagnosed with either DCM or AF at a heart test after the age of 8.5 years. Males were considered unaffected if they had reached the age of 6.5 years and had a heart test without a diagnosis of DCM or AF. There were a total of 72 individuals in the affected group, 29 of these had both DCM and AF diagnoses, 7 had a diagnosis of only DCM, and 36 had a diagnosis of only AF. A total of 23 individuals were ultimately included in the unaffected group, 9 females were unaffected by DCM or AF and were over the age of 8.5 years, and 14 males were unaffected by DCM or AF and were over the age of 6.5 years.

The number of individuals expected to develop DCM under four common penetrance models for each SNP was obtained by methods described by Camp (1997). The observed risk of disease for each genotype was obtained directly from the data by dividing the number of each genotype affected by the total number of individuals with the genotype. The genetic penetrance parameter (γ) was then established for each SNP. For the multiplicative, additive, and dominant models, γ indicates the relative risk of the heterozygote genotype compared to the homozygote genotype with the lowest risk, for the recessive model it indicates the relative risk of the most at risk homozygote compared to the least at risk homozygote. The penetrance parameter was then used to calculate the proportion of each genotype expected to develop disease under each model.

Table 2.4 shows how the penetrance parameter was used with the lowest homozygote risk under each model to calculate these proportions.

Table 2.4. The relationship of the risk of disease with the penetrance parameter.

	Penetrance		
Model	**a/a**	**a/A**	**A/A**
Multiplicative	f_0	$f_0\gamma$	$f_0\gamma^2$
Additive	f_0	$f_0\gamma$	$2f_0\gamma$
Recessive	f_0	f_0	$f_0\gamma$
Dominant	f_0	$f_0\gamma$	$f_0\gamma$

The risk of disease (f_0) of the least risky homozygote (a/a) is related to the genetic penetrance parameter (γ) for each additional genotype under multiplicative, additive, recessive, and dominant models. The penetrance parameter is a measure of the additional risk of disease that the risky allele confers to an individual. For the multiplicative, additive and dominant models γ is the relative risk of the heterozygote genotype compared to the homozygote genotype with the lowest risk, for the recessive model it is the relative risk of the most at risk homozygote compared to the least at risk homozygote.

The proportion of each genotype expected to develop disease under each model was then translated into the number expected by multiplying the proportion by the total number of individuals observed with the genotype, irrespective of disease status. A χ^2 test was performed to establish which model of penetrance most closely fitted the observed data. The model with the smallest χ^2 value was taken to be the best fit of the observed data.

Having established the model of penetrance, the most appropriate test for association with disease was then carried out. Where A is the disease associated allele, for dominant models the counts for individuals which genotyped as a/A and A/A can be pooled as the penetrance is equivalent, similarly for recessive models the counts for individuals genotyped as a/a and a/A can be pooled (Clarke et al., 2011). For multiplicative models allelic tests of association are more powerful (Clarke et al., 2011). Additive models are most appropriately tested by a Cochran-Armitage trend test, or genotype association testing (Clarke et al., 2011). To test for association with disease χ^2 tests were performed. The expected numbers of affected individuals with each genotype or allele were obtained by establishing the proportion of unaffected individuals with each genotype or allele. These proportions of unaffected individuals with each genotype or allele were then multiplied by the total number of affected

individuals to establish the expected number of affected individuals with each genotype. To account for multiple tests a Bonferroni correction was applied to the p values.

To establish whether particular genotypes gave an increased risk compared to the overall population risk, relative risks were calculated alongside 95% confidence intervals. Relative risks were calculated by dividing the genotype risk with the population risk and 95% confidence intervals were calculated using the methods in Morris and Gardner (1988). Where appropriate, genotypes were combined, for example where the penetrance model was shown to be recessive or dominant, or where there were less than 5 total individuals with a genotype.

To establish whether using multiple loci to test for an association could increase the association compared to individual loci alone, each significant locus was considered with each of the other significant loci in pairs and all combined. For this analysis the expected numbers were calculated and tested for an association in the same way as for loci individually. Risks of disease for each genotype combination were also established and relative risks calculated in the same way as individual loci.

2.8. Androgen receptor and *NCOA3* length associations with disease

2.8.1. Primers, PCR and fragment length analysis

The two polyglutamine repeat regions of the canine androgen receptor gene (Loci "*AR1*" and "*AR2*") were PCR amplified using the primers published by Maejima *et al.* (2005). Primers were also designed by Dr. N. Mongan to flank the polyglutamine region of the canine *NCOA3* gene. One of each pair of primers was fluorescently labelled with HEX or 6-FAM dyes (Sigma-Aldrich, USA) to allow fragment analysis to be carried out. Primer sequence information is in Table 2.5

Table 2.5. PCR primer sequences for androgen receptor loci *AR1* and *AR2*, and *NCOA3*.

Locus	Forward primer	Reverse primer
AR1	6-FAM-CCGTGAGCGCAGCACCTCCCGGTG	AGGCTGACCGCTGTTGGGAAGGCTGC
AR2	6-FAM-GCCAGCACCACCGGACGAGAATGA	TAACTGTCCTTGGAGGAGGTGGAAGCA
NCOA3	HEX-CCCAGCAGGGTTTTCTGAATGCCC	CACAGGCCCTGCCAAAACGCCATCC

Pre-PCR multiplexing whereby two PCR reactions are carried out on a sample in a single reaction was performed for *AR1* and *NCOA3* with no discernible effect on PCR efficiency. The reaction mixtures consisted of 1x LightCycler® 480 Probes Master Mix (Roche, Switzerland), primers at a concentration of 0.5µM, 1.5µL of template DNA per reaction, adjusted to 15µL using PCR grade H2O. The calculation for a 15µL reaction containing *AR1* and *NCOA3* primers combined was 7.5µL 2x Roche Probe Master Mix, 1.5µL of each primer from a stock with concentration of 5µM, and 1.5µL template DNA. *AR2* was added to the multiplex post PCR, the calculation for a 15µL reaction for the *AR2* primer pair was 7.5µL 2x Roche Probe Master Mix, 1.5µL of each primer from a stock with concentration of 5µM, 3µL H2O, and 1.5µL template DNA. PCR reactions were carried out in a Techne 512 or 412 thermo cycler (Bibby Scientific Limited, UK) under the following conditions: Initial denaturation 94°C for 30 seconds, 40 cycles of: 94°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 30 seconds, final extension 72°C for 5 minutes. The annealing temperature optimisation was determined by performing a temperature gradient PCR between 52°C and 64°C. An annealing temperature of 61°C was used for *AR1* and *NCOA3*, and 57°C was used for *AR2*.

All three fluorescently labelled PCR fragments were detected using an Applied Biosystems 3730 DNA Analyzer with GeneScan ROX-500 size standard (DBS Genomics, Durham, U.K.) in a single multiplex. This was possible because the FAM labelled PCR products were approximately 100bp different from each other. The multiplex consisted of the fluorescently labelled PCR fragments at a 1 in 100 dilution. Genotypes were scored using Genemapper software v3.7 (Applied Biosystems, USA). A homozygote of each allele was Sanger sequenced by Source Bioscience (Nottingham, UK) to confirm the number of polyglutamine repeats.

2.8.2. *AR* and *NCOA3* association with disease

The length of *AR1* and *AR2* were combined to give an overall *AR* polyglutamine repeat tract length for each individual. The *AR* gene is on the X chromosome, males only have one copy of the gene while females have two. For males the number of repeats in *AR1* and *AR2* were added together, while for females the mean of each was established and then added together. The mean length of *NCOA3* was established for all individuals. *t*-tests were performed using R statistical software (RCoreTeam, 2015) to establish whether there were differences between groups in the length of the polyglutamine repeat in *AR* and *NCOA3*. The groups that were compared were IWH DCM/AF (n = 59) vs not DCM/AF (n = 19), IWH osteosarcoma (n = 11) vs not osteosarcoma (n = 22), and Rottweiler osteosarcoma (n = 30) vs not osteosarcoma (n = 88). The individuals included in the unaffected ('not') groups were established by the age restrictions as determined above (Chapter 4). For each breed and diagnosis (IWH DCM/AF, IWH osteosarcoma, and Rottweiler osteosarcoma) the affected and unaffected groups were split by sex and ANOVAs were carried out to establish whether there were differences in the length of the polyglutamine tracts between the sexes in relation to disease development.

The frequency of the *AR1*, *AR2*, and *NCOA3* alleles was established utilising all genotyped individuals irrespective of disease status and χ^2 tests for each locus were performed to establish if there were significant differences between the breeds. The allele frequencies for each locus were visualised on bar charts by breed.

2.9. Fatty acid extraction and analysis

2.9.1. Samples

Remnants of whole blood samples taken from canine patients for veterinary diagnostic purposes were obtained and stored at -80°C (samples collected by cardiac specialist Professor Malcolm Cobb). Fatty acids were extracted from patients with a variety of diagnoses. These formed three patient groups: Group 1 (n = 10) dogs with DCM, Group 2 (n = 18) dogs with other heart diseases, and Group 3 (n = 4) dogs which had not been diagnosed with any form of

cardiac disease. The samples were age, sex and breed matched where possible. Details of the dogs are shown in Table 2.6.

Table 2.6. Diagnosis, age at diagnosis, sex and breed of all dogs used for fatty acid analysis.

Diagnosis	Age at diagnosis	Sex	Breed
DCM	Not available	M	Cocker Spaniel
DCM	6years 10months	FN	Great Dane
DCM	4years 3months	MN	Weimeraner
DCM	4years 10months	MN	Cocker Spaniel
DCM	7years 8 months	MN	Doberman
DCM	7years 6months	M	Boxer
DCM	11years 8months	MN	English Springer Spaniel
DCM	5years 3months	MN	Doberman
DCM	6years 3months	MN	Dogue de Bordeaux
DCM/end stage valve disease	6years 11months	ME	Deerhound
MVD and CHF	10years 3months	FN	Cavalier King Charles Spaniel
MVD	6years	M	Cavalier King Charles Spaniel
MVD	5years 4months	M	Cavalier King Charles Spaniel
MVD	15 years 6 months	FN	Cross breed
MVD	Not available	MN	Yorkshire Terrier
MVD	10years	ME	Cavalier King Charles Spaniel
MVD	7years 6months	M	Cavalier King Charles Spaniel
MVD	Not available	MN	Cavalier King Charles Spaniel
MVD	Not available	FN	Yorkshire Terrier
MVD	7years 5months	F	Cavalier King Charles Spaniel
MVD	8years 5months	ME	Bull Terrier
MVD	12years 6months	MN	Lhasa Apso
MVD and CHF	8years 7months	MN	English Springer Spaniel
MVD and CHF	Not available	M	Yorkshire Terrier
MVD and CHF	12years 4months	MN	Yorkshire Terrier
SAS	Not available	M	Boxer
TVD and AF	Not available	ME	Mastiff
valve disease and lung mass	8years	MN	Weimeraner
no cardiac diagnosis, aural haematoma	4years 8months	ME	Labrador
no cardiac diagnosis, RACL, skin disease	5years 2 months	MN	English Springer Spaniel
no cardiac diagnosis, myelofibrosis	7year 8months	FN	Boxer
no cardiac diagnosis, bone neoplasia	10years 5 months	MN	Collie

Diagnosis abbreviations: DCM – Dilated Cardiomyopathy, MVD – Mitral Valve Disease, TVD – Tricuspid Valve Disease, SAS – Subvalvular Aortic Stenosis, AF – Atrial Fibrillation, CHF – Congestive Heart Failure, RACL – Ruptured Anterior Cruciate Ligament. Sex is indicated by M for male and F for female with N indicating that the individual was neutered and E that it was entire, where N or E is absent this data was not available. Age at disease diagnosis is stated where this information was available.

2.9.2. Fatty acid extraction and methylation and gas chromatography

Fatty acids were extracted from 200µL whole blood using a modified Folch method (Folch et al., 1957). 750µL of cold chloroform:methanol (1:2) (Chloroform: Sigma-Aldrich, USA, Methanol: Thermo Fisher Scientific, USA) was vortexed (Topmix FB15012, Thermo Fisher Scientific, USA) for 1 minute with the blood sample in a borosilicate glass tube (KIMAX™, Kimble™, USA). To this 250µL cold chloroform and 250µL H_2O were added prior to vortexing for an additional 1 minute. Samples were then centrifuged (Sorvall™ Legend™ centrifuge, Thermo Fisher Scientific, USA) at 1,100x g for 15 minutes at 15°C. The organic chloroform phase containing the fatty acids was transferred to a clean borosilicate glass tube and dried under a nitrogen stream (Techne Sample Concentrator FDB03DD, Bibby Scientific Limited, UK). Samples were re-dissolved in 400µL hexane (Thermo Fisher Scientific, USA) prior to trans-esterification by the method of Christie (1982) modified by Chouinard *et al.* (1999), by vortexing 8µL of methyl acetate (Sigma-Aldrich, USA) and 8µL methylation reagent with samples. The methylation reagent consisted of 0.45mL of 30% sodium methoxide (Sigma-Aldrich, USA) and 2.05mL of methanol (Thermo Fisher Scientific, USA). This was allowed to react at room temperature for 10 minutes and then 12µL of termination reagent consisting of 0.1g of oxalic acid (Sigma-Aldrich, USA) in 3mL of diethyl ether (Sigma-Aldrich, USA) was added and briefly vortexed. 200mg of calcium chloride (Thermo Fisher Scientific, USA) was added to each sample, vortexed and incubated at room temperature for 1 hour. The samples were centrifuged for 1,100x g for 15 minutes at 15°C and the supernatant transferred to a gas chromatography vial for use directly in gas chromatography. Fatty acid isolation protocols and guidance were generously provided by Dr. Alison Mostyn, University of Nottingham who is gratefully acknowledged.

The fatty acid methyl esters were injected into a gas chromatograph (6890 Gas Chromatograph, Agilent, USA) by a split injection (ratio 50:1) with hydrogen used as the carrier gas. Separation of the fatty acid methyl esters was performed using a Varian CP-Sil 88 capillary column (Crawford Scientific Ltd, UK). The oven temperature was programmed to increase from 59°C to 100°C at a rate of 8°C per minute, it was then increased to 170°C at a rate of 6°C per

minute and held for 10 minutes. Following this the oven temperature was then increased to 240°C at a rate of 3°C per minute and held for 10 minutes. The temperature of the injector and detector were set at 255°C and 250°C respectively. The fatty acid methyl esters were identified by comparing the retention times with a fatty acid methyl esters standard mixture (Sigma-Aldrich, USA). The area percentage in moles of each fatty acid were used for the statistical analysis. The fatty acids analysed included saturated fatty acids: C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0, C24:0; monounsaturated fatty acids: C14:1, C15:1, C16:1, C17:1, C20:1, C24:1; polyunsaturated fatty acids: omega (n)-3: C18:3n3, C20:3n3, C20:5n3, C22:6n3, omega (n)-6: C18:2n6t, C18:2n6c, C18:3n6, C20:2n6, C20:3n6, C20:4n6, omega (n)-9: C18:1n9t, C18:1n9C, C22:1n9. The gas chromatography was completed by Dr Dongfang Li, School of Biosciences, University of Nottingham.

2.9.3. Fatty acid statistical analysis

The gas chromatography method quantifies an area value for each fatty acid. The relative abundance of each fatty acid in a sample was obtained by calculating the percentage that each fatty acid contributed to the overall profile of each sample. To establish which individuals clustered based on their fatty acid profiles, unsupervised hierarchical clustering analysis was performed using Cluster3.0 (de Hoon et al., 2004). The data was filtered so that there were at least four individuals with a measurement ≥0.1% for a gene (fatty acid). Fatty acids and arrays (samples) were mean-centred and hierarchical clustering of fatty acids and samples performed using average linkage. Fishers exact tests were performed using R statistical software (RCoreTeam, 2015) to establish whether there are differences in the number of individuals with DCM, valve disease, and no cardiac diagnosis between the two large clusters identified. This was based on the counts of individuals with each diagnosis type in each cluster. There were smaller clusters identified, but the sample size was not large enough to further test for differences between groups.

ANOVA tests were performed to establish whether there were differences between the percentages of any of the fatty acids between each of the groups

and a Bonferroni correction was applied to account for multiple tests. Following a significant ANOVA result, post-hoc Tukey tests were performed to establish which groups were significantly different from each other. ANOVAs and Tukey tests were carried out using R statistical software (RCoreTeam, 2015).

Where individual significant results were obtained for fatty acids that are related as biosynthetic precursors or products, these were then also considered together. The related fatty acids were plotted against each other on scatterplots per group and linear regression of each group was performed using R statistical software (RCoreTeam, 2015) to establish whether there were differences in the relationships between fatty acids between groups. The related fatty acids were also combined into a total percentage in each individual and ANOVAs and Tukey tests carried out using R statistical software (RCoreTeam, 2015) to test for differences in the combined percentage of these fatty acids.

The significant fatty acids that were related to each other as biosynthetic precursors or products from two separate fatty acid pathways. To test whether there was a relationship between pathway fatty acid percentages, the percentages of the significant fatty acids within each pathway were combined and plotted on a scatter plot with associated regression lines using R statistical software (RCoreTeam, 2015). The percentages from both pathways were combined into a single overall percentage for each individual for determination of ANOVA.

Chapter 3 A predictive model for canine dilated cardiomyopathy – a meta-analysis of Doberman Pinscher data

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PeerJ

A predictive model for canine dilated cardiomyopathy—a meta-analysis of Doberman Pinscher data

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ABSTRACT

Dilated cardiomyopathy is a prevalent and often fatal disease in humans and dogs. Indeed dilated cardiomyopathy is the third most common form of cardiac disease in humans, reported to affect approximately 36 individuals per 100,000 individuals. In dogs, dilated cardiomyopathy is the second most common cardiac disease and is most prevalent in the Irish Wolfhound, Doberman Pinscher and Newfoundland breeds. Dilated cardiomyopathy is characterised by ventricular chamber enlargement and systolic dysfunction which often leads to congestive heart failure. Although multiple human loci have been implicated in the pathogenesis of dilated cardiomyopathy, the identified variants are typically associated with rare monogenic forms of dilated cardiomyopathy. The potential for multigenic interactions contributing to human dilated cardiomyopathy remains poorly understood. Consistent with this, several known human dilated cardiomyopathy loci have been excluded as common causes of canine dilated cardiomyopathy, although canine dilated cardiomyopathy resembles the human disease functionally. This suggests additional genetic factors contribute to the dilated cardiomyopathy phenotype.This study represents a meta-analysis of available canine dilated cardiomyopathy genetic datasets with the goal of determining potential multigenic interactions relating the sex chromosome genotype (XX vs. XY) with known dilated cardiomyopathy associated loci on chromosome 5 and the PDK4 gene in the incidence and progression of dilated cardiomyopathy. The results show an interaction between known canine dilated cardiomyopathy loci and an unknown X-linked locus. Our study is the first to test a multigenic contribution to dilated cardiomyopathy and suggest a genetic basis for the known sex-disparity in dilated cardiomyopathy outcomes.

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INTRODUCTION

Dilated cardiomyopathy (DCM) is a prevalent and often fatal disease requiring clinical management in humans and dogs (*Egenvall, Bonnett & Häggström, 2006*; *Hershberger, Morales & Siegfried, 2010*). DCM is the second most common cardiac disease in dogs and is characterised by ventricular chamber enlargement and systolic dysfunction which often

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Author contribution

Siobhan Simpson conceived and designed the experiments, performed the majority of the experiments, analysed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and tables, reviewed drafts of the paper.

Jennifer Edwards and Richard D. Emes performed the experiments, analysed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Malcolm A. Cobb analysed the data, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper, funding.

Nigel P. Mongan and Catrin S. Rutland conceived and designed the experiments, performed the experiments, analysed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper, funding.

3.1. Abstract

Dilated cardiomyopathy is a prevalent and often fatal disease in humans and dogs. Indeed dilated cardiomyopathy is the third most common form of cardiac disease in humans, reported to affect approximately 36 individuals per 100,000 individuals. In dogs, dilated cardiomyopathy is the second most common cardiac disease and is most prevalent in the Irish Wolfhound, Doberman Pinscher and Newfoundland breeds. Dilated cardiomyopathy is characterised by ventricular chamber enlargement and systolic dysfunction which often leads to congestive heart failure. Although multiple human loci have been implicated in the pathogenesis of dilated cardiomyopathy, the identified variants are typically associated with rare monogenic forms of dilated cardiomyopathy. The potential for multigenic interactions contributing to human dilated cardiomyopathy remains poorly understood. Consistent with this, several known human dilated cardiomyopathy loci have been excluded as common causes of canine dilated cardiomyopathy, although canine dilated cardiomyopathy resembles the human disease functionally. This suggests additional genetic factors contribute to the dilated cardiomyopathy phenotype.This study represents a meta-analysis of available canine dilated cardiomyopathy genetic datasets with the goal of determining potential multigenic interactions relating the sex chromosome genotype (XX vs. XY) with known dilated cardiomyopathy associated loci on chromosome 5 and the *PDK4* gene in the incidence and progression of dilated cardiomyopathy. The results show an interaction between known canine dilated cardiomyopathy loci and an unknown X-linked locus. Our study is the first to test a multigenic contribution to dilated cardiomyopathy and suggest a genetic basis for the known sex-disparity in dilated cardiomyopathy outcomes.

3.2. Introduction

Dilated cardiomyopathy (DCM) is a prevalent and often fatal disease requiring clinical management in humans and dogs (Egenvall et al., 2006, Hershberger et al., 2010a). DCM is the second most common cardiac disease in dogs and is characterised by ventricular chamber enlargement and systolic dysfunction which often leads to congestive heart failure (Egenvall et al., 2006). The aetiology of DCM is complex. Genetic factors, myocardial ischemia,

hypertension, toxins, infections and metabolic defects have been implicated (McNally et al., 2013). To date, mutations in over 50 genes have been associated with DCM in humans; however, mutations in the most prevalent DCM related genes only account for approximately 50% of patients with DCM (Posafalvi et al., 2012). In human DCM genetic testing where a panel of approximately 50 loci are tested concurrently, often more than one locus can be implicated in the disease (McNally et al., 2013), suggesting multiple genetic factors cooperate in DCM aetiology.

Canine DCM is phenotypically similar to human DCM (Shinbane et al., 1997). As outlined below, to date mutations in only two genes (*PDK4* and *STRN*) and a single nucleotide polymorphism (SNP) on chromosome 5 have been associated with canine DCM (Mausberg et al., 2011, Meurs et al., 2012, Meurs et al., 2013), suggesting additional genetic causes remain unknown. While canine studies have sometimes been limited by small sample size (typically less than 10 individuals), those studies with larger sample numbers (greater than 50 individuals) have also frequently failed to find significant associations with DCM (e.g., (Philipp et al., 2007, Philipp et al., 2008b, Wiersma et al., 2008)). One possible explanation for the challenges in identifying DCM associated loci in humans and dogs is that even within an extended family or breed, genetic variation at a single locus cannot explain the development of DCM. Indeed dog breeds can be considered as large families, with dogs within a breed more related to each other than dogs of other breeds (Parker et al., 2004). In the same way that some human families are affected by DCM, a subset of dog breeds are affected by DCM more frequently than others (Egenvall et al., 2006). Dobermans Pinschers (hereafter Dobermans) are particularly affected by DCM, with both a high prevalence (58.2% in European Dobermans) and severity with DCM associated death often occurring within 8 weeks of diagnosis (Calvert et al., 1997, Wess et al., 2010). In dogs, diagnosis is usually at the onset of clinical symptoms of heart failure. But there is an extended pre-clinical phase, during which treatment can be effective by prolonging the onset of heart failure (Summerfield et al., 2012). In this phase left ventricular dilation and dysfunction begins, and can be accompanied by ventricular premature complexes (Singletary et al., 2012), Median life

expectancy of DCM affected European Dobermans is 7.8 years, compared with 11 years for unaffected European Dobermans (Egenvall et al., 2006, Proschowsky et al., 2003). A deletion in a splice site of the *PDK4* gene (Meurs et al., 2012) and a SNP on chromosome 5 (Mausberg et al., 2011) in Dobermans are two of only three canine DCM mutations identified to date. While these two loci are associated with Doberman DCM, individually neither locus explains all cases of Doberman DCM (Mausberg et al., 2011, Meurs et al., 2012). Individuals heterozygous at the Chr5 SNP are more likely to develop DCM, but there are many DCM cases homozygous for the healthy allele (Mausberg et al., 2011). While *PDK4* genotypes are less definite predictors of DCM, with both affected and unaffected individuals possessing the three possible genotypes, the 16bp *PDK4* splice site deletion is found more frequently in North American Dobermans with DCM than those without DCM (Meurs et al., 2012). However an analysis of European Dobermans failed to identify an association between *PDK4* and DCM (Owczarek-Lipska et al., 2013), suggesting additional unknown factors influence the effect of *PDK4* in predisposing individuals to DCM. Thus novel genetic causes of canine DCM remain to be identified (Mausberg et al., 2011, Philipp et al., 2012).

In this study we developed genetic models to test the influence of unknown genetic factors to predict which DCM-associated genotype combinations are likely to develop DCM. Using this method, we provide evidence for a sex-linked genetic influence on known DCM loci in the pathogenesis of canine DCM. Our study is the first to propose a multigenic contribution to canine DCM.

3.3. Methods

3.3.1. Model development

A literature search of the Pubmed and Web of Science databases using the following search terms: “Doberman DCM loci”, “Doberman Dilated Cardiomyopathy loci”, “Doberman DCM gene”, “Doberman Dilated Cardiomyopathy gene”, “Doberman DCM locus”, and “Doberman Dilated Cardiomyopathy locus” (Figure 3.1) identified thirty unique records. This search identified two loci associated with DCM in North American Dobermans (Mausberg et al., 2011, Meurs et al., 2012). By combining the genotypes from

the identified Doberman DCM associated loci, and additional putative loci, predictive models were developed and tested against observed DCM incidence data. All genotype combinations for the DCM associated SNP identified on chromosome 5 (TIGRP2P73097:CFA5:g.53,941,386T>C, CanFam2.1) (Mausberg et al., 2011) and the *PDK4* (GeneID:482310) splice site deletion (CFA14:g.20,829,667_20,829,682del, CanFam3.1) (Meurs et al., 2012) were determined. Further analysis determined which genotype combinations were likely to lead to DCM. Some genotypes are definitive; all individuals homozygous for the susceptibility allele at CFA5:g.53,941,386T>C develop DCM (Mausberg et al., 2011).

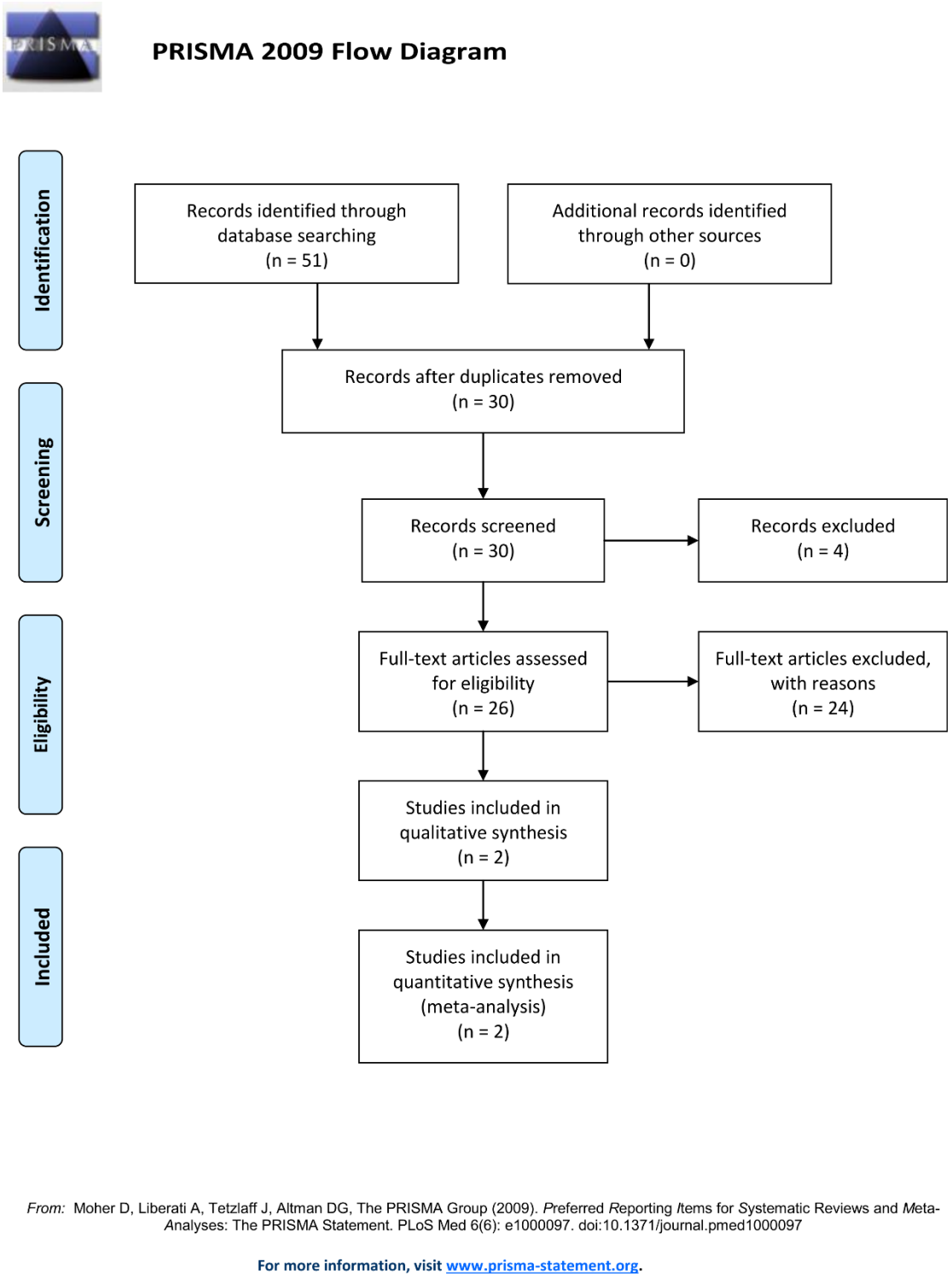


Figure 3.1. Prisma flow diagram. DOI: 10.7717/peerj.842/fig-1 Shows how many records were found in the literature search, where records were removed such as duplicates, subsequent exclusion due to not being fully published, for example conference abstracts. Additional articles were then excluded as they did not identify loci associated with DCM in Dobermans.

3.3.2. Determining which genotypes develop DCM

Five genetic models incorporating genotypes at multiple observed and hypothetical loci were developed including: 1. two known DCM loci; 2. two known loci + 50% of the population more susceptible to developing DCM; 3. two known loci + a novel autosomal dominant DCM locus; 4. two known loci + a novel autosomal recessive DCM locus; 5. two known loci + a novel additive DCM locus and 6. two known loci + a novel X-linked DCM locus. For each model, different biologically feasible phenotype outcomes were tested for each genotype combination to establish the best fit of the model to the observed DCM incidence data. Each model was subject to the following constraints: individuals that are homozygous CC at the Chr5 SNP develop DCM, and individuals with no susceptibility alleles are healthy.

3.3.3. Model testing

For each model, the frequency of each genotype combination was calculated by multiplying the genotype frequencies using *PDK4* and Chr5 frequencies (Table 3.1) obtained from Owczarek-Lipska *et al.* (2013) and Mausberg *et al.* (2011). A range of frequencies were tested for each hypothetical loci. For example, for the model incorporating only *PDK4* and Chr5 variants, one genotype combination is WtWt-TT. The frequency of this genotype combination is the product of the frequency of WtWt and the frequency of TT in the population. From the combined genotype frequencies, the expected numbers of individuals with each genotype combination were calculated by multiplying the frequency by the number of individuals in the study to be compared with (182 when compared with Mausberg *et al.* (2011) and Owczarek-Lipska *et al.* (2013)). Thus, the numbers of individuals in the model that were, for example, WtWt healthy and WtWt DCM were obtained by summing the numbers in each category. Having obtained the numbers of affected and unaffected individuals that the model predicts for each genotype, these were tested against the observed data using a χ^2 test. Where additional putative DCM loci were included in the model, several allele frequencies were tested. However, as GWAS studies have previously been carried out (Mausberg et al., 2011, Meurs et al., 2012), it is unlikely that additional DCM alleles are at higher frequencies than those already identified. For this reason, DCM allele frequencies over 0.5

were not tested. If the model is a good fit of the observed data, the χ^2 test statistic will be non-significant.

Table 3.1. Genotype frequencies assuming Hardy Weinberg equilibrium.

PDK4		**Chr5 SNP**	
Genotype	**Freq**	**Genotype**	**Freq**
Wt Wt	0.72	TT	0.74
Wt del	0.26	TC	0.24
Del del	0.02	CC	0.02

Allele frequencies taken from Mausberg *et al.* (2011) and Owczarek-Lipska *et al.* (2013). DOI: 10.7717/peerj.842/table-1

The proportion of the population that the model predicts to have DCM was determined by taking the sum of all the genotype combined frequencies that lead to DCM in the model. For example, for the model incorporating just the two known loci this is 0.0144 + 0.0624 + 0.0052 + 0.0048 + 0.0004 = 0.0872 – (Appendix 5, Supplementary Table 1). This proportion was then compared to the observed DCM frequency of 0.582 (Wess et al., 2010).

For most models, it must be assumed that there is no difference in DCM incidence between the sexes, as an effect of sex has not been included. For the DCM model testing a 50% increased susceptibility, where it is biologically feasible that males are more susceptible and the models incorporate an additional X-linked locus, it is possible to calculate the proportion males and females that develop DCM. While males develop clinical symptoms earlier and appear to be more severely affected, there are indications that the sex of those affected by DCM is close to 50% male, 50% female (Wess et al., 2010), so we would expect our model to reflect this.

Odds ratios of each genotype and allele developing DCM for each model were obtained by testing each genotype against the other two combined and each allele against the other. Odds ratios are the odds/probability of an individual with a particular genotype or allele developing DCM compared, by dividing one by the other, to the odds of an individual with all other genotypes or alleles developing DCM, with an odds ratio greater than one associated with the trait of interest and an odds ratio of less than one not associated (Bland and Altman,

2000). For example the odds ratio for TT in the published data from Mausberg *et al.* (2011) is calculated in the following way. There are 45 individuals that are TT DCM and 85 TT healthy the odds of a TT individual developing DCM are 45/85 (0.53), there are 43 individuals which are TC or CC with DCM and 9 individuals that are TC or CC healthy so the odds of these individuals developing DCM are 43/9 (4.78) the odds ratio divides the genotype of interest odds by the 'others' odds to give the odds ratio or 0.11. To assess the significance of these ratios χ^2 tests were performed on the 2 × 2 tables—in the above example the four groups are TT-DCM, TT-healthy, TC or CC-DCM, TC or CC-healthy. If the model is a good fit to the observed data it is expected that the odds ratios are of a similar pattern and significance, e.g., TT, small—significantly not associated with DCM; TC, large—significantly associated with DCM; CC, not possible to test—not testable, as for the Chr5 SNP in Table 3.2. Odds ratios of both genotypes and alleles were obtained from the original studies (Table 3.2 and 3.3).

Table 3.2. Genotype odds ratios from the original studies reporting an association.

Genotype	Odds ratio	95% CI
PDK4 WtWt	0.14	0.07, 0.32
PDK4 WtDel	5.21	2.70, 12.09
PDK4 DelDel	1.14	0.41, 3.18
Chr5 TT	0.11	0.05, 0.24
Chr5 TC	6.23	2.78, 14.00
Chr5 CC	NA	NA

Ratios from the *PDK4* locus (Meurs et al., 2012) and Chromosome 5 SNP (Mausberg et al., 2011). The *PDK4* χ^2 test results indicate that the WtWt genotype significantly associated with non-DCM and the WtDel genotype significantly associated with DCM at the 0.01 significance level, the DelDel genotype odds ratio whilst different from the null result of 1, is not significantly so. For the chromosome 5 SNP all individuals that are CC in the original study developed DCM, thus and odds ratio and confidence interval cannot be calculated, but χ^2 tests can be performed on the data. TT is significantly associated with non-DCM and the TC and CC genotypes are significantly associated with DCM at the 0.01 significance level. DOI: 10.7717/peerj.842/table-2

Table 3.3. Allele odds ratios from the original studies reporting DCM associations.

Allele	Odds ratio	95% CI
PDK4 Wt	0.38	0.23, 0.64
PDK4 Del	2.63	1.57, 4.42
Chr5 T	0.12	0.06, 0.26
Chr5 C	8.11	3.85, 17.09

Allele odds rations at the *PDK4* locus (Meurs et al., 2012) and Chromosome 5 SNP (Mausberg et al., 2011). The χ^2 test results indicate that each susceptibility (Del and C respectively) allele is significantly associated with DCM and the alternate allele significantly associated with non-DCM at the 0.01 significance level. DOI: 10.7717/peerj.842/table-3

3.4. Results

Following the constraints stated in the methods and using biologically feasible reasoning each model was optimised to best fit the observed data. For each model the genotype-phenotype decision descriptions are shown in Table 3.4. Tables of each model are in Appendix 5.

3.4.1. Comparing model predictions with observed data

The χ^2 test values comparing predicted numbers with observed numbers of DCM and healthy individuals at each genotype ranged from 4.35 to 7766.06. A χ^2 value of less than 11.07 indicates there is no significant difference between predicted and observed genotype-phenotype data, (5% significance level, with 5 degrees of freedom). Values less than 15.09 represent predictions not significantly different to observed values at the 1% significance level. χ^2 values less than these critical values are indicated in Table 3.5.

3.4.2. Model predicted DCM population frequency and sex incidence

For each model, the predicted DCM frequency was calculated to provide an additional method to test the accuracy of the model. The DCM frequency in the European Doberman population is estimated to be 58.2% (Wess et al., 2010), therefore accurate models should predict a similar frequency. The frequencies predicted by each model are displayed in Table 3.6 (see also Appendix 5, Supplementary Table 2), with those within 10% of the reported frequency highlighted as accurate models. Further to this the proportion of males and

females that each model predicts to develop DCM were calculated. Whilst most models do not account for sex and assume equal numbers of males and females affected, two models tested either a 50% increase in male susceptibility or an additional X-linked locus. Based on reported DCM incidence for a model to fit the observed data it is expected that similar proportions of males and females develop DCM. Table 3.7 shows that irrespective of the frequency of the novel susceptibility allele the model incorporating a novel X linked DCM locus gives similar proportions of affected males and females.

Table 3.4. Genotype-phenotype decision descriptions for each model.

Model	Genotype—phenotype decision description, in addition to the rules:
1.	DCM develops when both the *PDK4* locus and Chr5 SNP have at least one DCM susceptibility allele.
2.	50% more susceptible only need to have a single DCM susceptibility allele at either locus to develop DCM while the 50% less susceptible to DCM require at least one DCM susceptibility allele at both loci to develop DCM.
3.	All individuals that have a susceptibility allele at the additional locus develop DCM. Those individuals with no susceptibility alleles at the additional locus need at least one DCM susceptibility allele at both of the other loci to develop DCM.
4.	All homozygous susceptible individuals at the additional locus develop DCM. For individuals that are heterozygous at the additional locus, DCM occurs when combined with another DCM susceptibility allele, while homozygous unsusceptible individuals need at least one DCM susceptibility allele at both of the other loci to develop DCM.
5.	All homozygous susceptible individuals at the additional locus develop DCM. Heterozygotes and homozygous unsusceptible individuals need at least one DCM susceptibility allele at both of the other loci to develop DCM.
6.	X linked susceptible DCM locus males can either possess a single unsusceptible X (XY) or a single susceptible x (xY), while females can be unsusceptible X homozygotes (XX), heterozygotes (Xx) or susceptible x homozygotes (xx). Unsusceptible X males (XY) are phenotypically identical to unsusceptible X homozygotes (XX) with these individuals requiring at least one DCM susceptibility allele at both of the other loci to develop DCM. All individuals that possess a susceptible X (xY and xx individuals) develop DCM in this model while heterozygotes (Xx) only require a single DCM susceptibility allele at one of the other loci to develop DCM.

Models represent: 1. the two known DCM loci; 2. two known loci + 50% of the population is more susceptible to developing DCM; 3. two known DCM loci combined with a novel autosomal dominant DCM locus; 4. two known DCM loci combined with an autosomal recessive locus; 5. two known DCM loci combined with a an additional DCM locus that is additive and 6. two known DCM loci combined with an X-linked DCM locus. DOI: 10.7717/peerj.842/table-4

Table 3.5. χ^2 test statistic results comparing predicted of DCM and healthy individuals at each genotype from each model with observed numbers of DCM and healthy individuals at each genotype.

	χ2 test statistic for each model							
Model	***PDK4***				**Chr5**			
1.	1269.23				7766.06			
2.	110.45				596.68			
DCM allele freq	**0.5**	**0.4**	**0.3**	**0.2**	**0.5**	**0.4**	**0.3**	**0.2**
3.	32.47	29.25	51.42	113.35	6.58**	7.69**	24.30	69.27
4.	26.24	74.61	171.69	379.06	31.65	67.45	145.76	360.86
5.	88.95	31.36	4.97**	4.36**	114.72	53.10	23.13	17.21
DCM X allele (x) freq	**0.5**	**0.4**	**0.3**	**0.2**	**0.5**	**0.4**	**0.3**	**0.2**
6.	10.57**	10.06**	25.38	71.30	11.32*	9.29**	19.55	52.86

Model data based on data from Mausberg *et al.* (2011) – Chr5 SNP and Owczarek-Lipska *et al.* (2013) – *PDK4*. * not significant at 1% significance level. ** not significant at 5% significance level. DOI: 10.7717/peerj.842/table-5

Table 3.6. DCM frequency predicted by each model.

Model	**DCM freq for each model**			
1.	0.0872			
2.	0.2772			
DCM allele freq	**0.5**	**0.4**	**0.3**	**0.2**
3.	0.5054*	0.415648	0.328952	0.245321
4.	0.3154	0.233248	0.169352	0.123712
5.	0.7718	0.671392*	0.552728*	0.415808
DCM X allele (x) freq	**0.5**	**0.4**	**0.3**	**0.2**
6.	0.5245*	0.433984	0.350432	0.257536

* indicates frequencies within 0.1 of the reported frequency (0.582 (Wess et al., 2010)) in the European Doberman pincher population. DOI: 10.7717/peerj.842/table-6

Table 3.7. Proportion of males and females predicted to be affected by DCM by models 2 and 6.

	Proportion DCM	
Model	**Male**	**Female**
2.	0.4672	0.0872
6.		
DCM X allele (x) freq 0.5	0.5436	0.5054
DCM X allele (x) freq 0.4	0.45232	0.415648
DCM X allele (x) freq 0.3	0.36104	0.339824
DCM X allele (x) freq 0.2	0.26976	0.245312

DOI: 10.7717/peerj.842/table-7

3.4.3. Odds ratios

For the Chr5 SNP there are no odds ratio for CC, as all individuals that are CC develop DCM in both the original study (Mausberg et al., 2011) and models so odds ratios cannot be calculated. Despite this a χ^2 test can be performed on the counts of affected and unaffected individuals observed and predicted with the genotype so the significance of the results was obtained. For the Chr5 SNP, 12 of 18 models (Table 3.9), and 15 of the allele odds ratios are consistent with the original studies (Table 3.11). The *PDK4* deletion association was identified in the North American Doberman population; in the European population, the odds ratios (Table 3.8 and 3.10) are not significantly different from the null result of 1. Once combined with additional loci, similar significant likelihood ratios as the North American population are obtained for 13 of 18 models (Table 3.8 and 3.10).

3.4.4. Selecting the most realistic model

For a model to be considered plausible, it should predict similar numbers of affected and unaffected individuals at each genotype as observed in Mausberg *et al.* (2011) and Owczarek-Lipska *et al.* (2013), predict similar DCM frequency as reported in the population (Wess et al., 2010), and give odds ratios of genotypes and alleles similar to those from the studies which report an association. To assist in determining which models meet these requirements, Table 3.12 shows which conditions each model meets (Appendix 5, Supplementary Tables 3 – 6). From this it is possible to see that no model meets all the conditions, but two similar models, the models incorporating the two identified loci and an additional X-linked DCM locus with the novel DCM allele frequency at 0.4 and 0.5, meet all but one condition each. An additional exploration of the additional X-linked DCM allele frequency indicates that an X-linked DCM allele frequency between 0.4 and 0.5 leads to all conditions being met.

Table 3.8. Odds ratios of each *PDK4* genotype with χ^2 significance.

	***PDK4* genotype odds ratio**											
Model	**wtwt**	**wtdel**	**deldel**	**wtwt**	**wtdel**	**deldel**	**wtwt**	**wtdel**	**deldel**	**wtwt**	**wtdel**	**deldel**
Individual loci	0.78	1.29	1.11									
1.	0.06**	12.91**	3.85									
2.	0.1**	9.41**	4.6									
DCM allele freq	**0.5**			**0.4**			**0.3**			**0.2**		
3.	0.14**	6.70**	4.42	0.15**	6.31**	3.98	0.15**	6.21**	3.69	0.14**	6.47**	3.53
4.	0.45*	2.17*	1.76	0.35**	2.73**	2.03	0.25**	3.77**	2.43	0.15**	5.82**	2.98
5.	0.7	1.42	1.31	0.67	1.49	1.36	0.62	1.6	1.43	0.53	1.84	1.58
DCM X allele (x) freq	**0.5**			**0.4**			**0.3**			**0.2**		
6.	0.31**	3.12**	2.4	0.30**	3.23**	2.41	0.28**	3.41**	2.45	0.24**	3.89**	2.59

* significant at 5% level, ** significant at 1% level. DOI: 10.7717/peerj.842/table-8

Table 3.9. Odds ratios of each Chr5 SNP genotype with χ^2 significance.

	Chr5 genotype odds ratio											
Model	**TT**	**TC**	**CC**	**TT**	**TC**	**CC**	**TT**	**TC**	**CC**	**TT**	**TC**	**CC**
Individual loci	0.11**	6.23**	–**									
1.	0.02**	11.37**	–**									
2.	0.09**	9.23**	–**									
DCM allele freq	**0.5**			**0.4**			**0.3**			**0.2**		
3.	0.14**	6.74**	–	0.14**	6.34**	–*	0.13**	6.25**	–**	0.12**	6.56**	–**
4.	0.35**	2.33*	–**	0.25**	2.96**	–**	0.16**	4.13**	–**	0.08**	6.45**	–**
5.	0.67	1.51	–	0.61	1.57	–	0.54	1.7	–	0.44*	1.96	–*
DCM X allele (x) freq	**0.5**			**0.4**			**0.3**			**0.2**		
6.	0.29**	3.22**	–	0.27**	3.34**	–*	0.24**	3.55**	–**	0.19**	4.08**	–**

* significant at 5% level, ** significant at 1% level. DOI: 10.7717/peerj.842/table-9

Table 3.10. Odds ratios of each PDK4 allele with χ^2 significance.

	***PDK4* allele odds ratio**							
Model	**Wt**	**Del**	**Wt**	**Del**	**Wt**	**Del**	**Wt**	**Del**
Individual loci	0.81	1.23						
1.	0.17**	5.84**						
2.	0.16**	6.22**						
DCM allele freq	**0.5**		**0.4**		**0.3**		**0.2**	
3.	0.19**	5.37**	0.2**	4.91**	0.22**	4.65**	0.22**	4.57**
4.	0.52*	1.94*	0.43**	2.32**	0.34**	2.94**	0.22**	3.91**
5.	0.74	1.36	0.71	1.36	0.66	1.51	0.59	1.69
DCM X allele (x) freq	**0.5**		**0.4**		**0.3**		**0.2**	
6.	0.37**	2.71**	0.36**	2.76**	0.35**	2.94**	0.32**	3.1**

* significant at 5% level, ** significant at 1% level. DOI: 10.7717/peerj.842/table-10

Table 3.11. Odds ratios of each Chr5 SNP allele with χ^2 significance.

	Chr5 allele odds ratio							
Model	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**
Individual loci	0.15**	6.64**						
1.	0.08**	12.33**						
2.	0.13**	7.49**						
DCM allele freq	**0.5**		**0.4**		**0.3**		**0.2**	
3.	0.19**	5.34**	0.19**	5.37**	0.18**	5.55**	0.16**	6.07**
4.	0.36**	2.76**	0.28**	3.62**	0.20**	5.08**	0.16**	7.68**
5.	0.72	1.38	0.64	1.38	0.55	1.82	0.45**	2.23**
DCM X allele (x) freq	**0.5**		**0.4**		**0.3**		**0.2**	
6.	0.33**	3.02**	0.3**	3.28**	0.27**	5.08**	0.23**	4.35**

* significant at 5% level, ** significant at 1% level. DOI: 10.7717/peerj.842/table-11

Table 3.12. Adherence to model.

	χ^2			OR genotype		OR allele		Number of
Model	*PDK4*	Chr5 SNP	DCM freq	*PDK4*	Chr5 SNP	*PDK4*	Chr5 SNP	conditions not met
Individual	–	–	–	x	Y	x	x	3
1.	x	x	x	Y	Y	Y	x	4
2.	x	x	x	Y	Y	Y	x	4
3.								
0.5	x	x	Y	Y	x	Y	Y	3
0.4	x	x	x	Y	Y	Y	Y	3
0.3	x	x	x	Y	Y	Y	Y	3
0.2	x	x	x	Y	Y	Y	Y	3
4.								
0.5	x	x	x	Y	Y	Y	Y	3
0.4	x	x	x	Y	Y	Y	Y	3
0.3	x	x	x	Y	Y	Y	Y	3
0.2	x	x	x	Y	Y	Y	Y	3
5.								
0.5	x	x	x	x	x	x	x	7
0.4	x	x	Y	x	x	x	x	6
0.3	Y	x	Y	x	x	x	x	5
0.2	Y	x	x	x	x	x	Y	5
6.								
0.5	Y	Y	Y	Y	x	Y	Y	1
0.4	Y	Y	x	Y	Y	Y	Y	1
0.3	x	x	x	Y	Y	Y	Y	3
0.2	x	x	x	Y	Y	Y	Y	3

Table shows whether each model (with the new DCM allele frequency indicated) meets each condition, Y the condition is met, x the condition is not met. The number of conditions not met is also indicated. DOI: 10.7717/peerj.842/table-12

3.5. Discussion

This study used publicly available data to test the prediction that genetic models incorporating multiple factors can better explain and predict the incidence of canine DCM than those utilising a single factor. Until now, the possibility that multiple genes combine to influence DCM phenotype has been proposed, but has not yet been tested, despite an established role for multiple loci in related diseases (Ingles et al., 2005, Posafalvi et al., 2012, Rampersaud et al., 2011, Xu et al., 2010). This is the first study to investigate the combined effect of multiple factors on the predisposition to DCM. Although our models do not explain all cases of canine DCM, by combining three factors (*PDK4*, Chr5 TIGRP2P73097 SNP and an X-linked locus) we show that DCM incidence can be more accurately predicted (Tables 3.6 – 3.12). Furthermore, as noted above the PDK4 splice site deletion is not significantly associated with DCM in the European population. But in the model incorporating only the two known loci,

the *PDK4* variant improves the odds ratio for the Chr5 SNP. Collectively these findings indicate that models incorporating multiple factors are more effective than those incorporating a single factor. This result is important because it has implications for future studies of the genetics and management of DCM. A better understanding of the genetic basis of DCM will permit the monitoring and earlier clinical intervention of high risk individuals thus potentially improving the outcome for affected individuals.

To assess the accuracy of each model, we performed several statistical tests. For any model to be considered an accurate representation of observed data it should predict similar numbers of affected and unaffected individuals at each genotype as have been reported in the published data. It should also predict a similar DCM frequency to that found in the population. Secondly, the odds ratios of genotypes and alleles should support an association of the specific variants with DCM. The models incorporating the two known DCM loci and an additional X-linked locus with a susceptible allele frequency of 0.46 for the novel susceptible allele met all such conditions. It is important to note that this susceptible allele frequency should have been identified by the previous GWAS studies (Mausberg et al., 2011, Meurs et al., 2012). It is therefore possible that additional cases and controls are required to complete a comprehensive GWAS analysis of DCM in Dobermans to establish the function and frequency of this predicted DCM associated locus.

Most predictive models are based on either known or simulated genotypes at multiple loci (Janssens et al., 2006, Pencina et al., 2008). Such models do not account for known effects of genotypes or allow the inclusion of additional as yet unknown, loci. For example, in this study all individuals possessing the Chr5 CC genotype have DCM. Our methodology is unique and useful where there are multiple known and unknown factors which do not fully account for the phenotype. In particular, our approach accommodates specific gene combinations to lead to disease, rather than incremental risk factors as is the case in other predictive models (Janssens et al., 2006, Pencina et al., 2008). Limitations to our methodology include the number of factors that can be modelled is limited by the available data. Despite this, our methodology could

be used in other situations. While many phenotypes are the consequence of multiple loci, there can be some loci which have comparatively more important contribution to the phenotype (e.g., (Papa et al., 2013, Strange et al., 2011)). Identifying these loci can be the first steps in predicting phenotypes (e.g., (Hayes et al., 2010, Papa et al., 2013)). Following the identification of loci associated with a trait, our methodology can be used to indicate what type of additional loci may be influencing the trait of interest, which may simplify the identification of additional loci.

3.6. Conclusions

There are many unknown factors involved in the aetiology of canine and human DCM. In Dobermans, we have identified multigenic effects and a possible X-linked locus as novel variables influencing DCM risk. While the *PDK4* splice site deletion and the Chr5 SNP have both been tested for association with DCM in the European population of Dobermans, the combined genotype of individuals has not yet been considered (Mausberg et al., 2011, Owczarek-Lipska et al., 2013). Our model would benefit from further genotyping of Dobermans at both the *PDK4* and Chr5 variants to further validate the model. Future work is also required to identify X-linked DCM loci if the model is verified for the known loci. It is also possible that the different combinations of alleles leading to DCM in the model could affect the time taken to progress from one disease stage to the next as reported by Wess *et al.* (2010). If validated, our model has implications for current canine breeding practices and welfare of individuals within the breed. Individuals with allele combinations more likely to develop DCM can be monitored more intensely than those with less genetic risk, and mating pairs resulting in deleterious genotypes can be avoided. This will improve welfare by reducing the prevalence of DCM-associated alleles within the population and potentially improving the longevity of affected dogs by enabling monitoring and earlier clinical management. By utilising similar methodology, equivalent multigenic effects and possible additional loci could be identified in human DCM, giving similar benefits to those described for Dobermans.

Chapter 4 Multiple genetic associations with Irish Wolfhound Dilated Cardiomyopathy

This chapter is prepared for publication and is currently under review at BioMed Research International for the special issue “Genetic and Epigenetic Regulation Networks: Governing from Cardiovascular Development to Remodelling” so is presented in paper format.

4.1. Abstract

Cardiac disease is a leading cause of morbidity and mortality in both humans and dogs, with dilated cardiomyopathy a large contributor to this. Dilated cardiomyopathy is reported to affect 36 per 100,000 people and is one of the most common causes of canine cardiac disease. Mutations in over 50 genes are associated with human dilated cardiomyopathy, but very few associated with canine dilated cardiomyopathy. The Irish Wolfhound is one of the most commonly affected breeds and as such it is one of the few breeds in which genetic loci associated with the disease have been identified. None of the previously identified canine loci explain many cases of the disease in the breed and previous work has indicated that genotypes at multiple loci may act together to influence dilated cardiomyopathy development. In this study, loci previously identified as associated with Irish Wolfhound dilated cardiomyopathy were tested for associations in a new cohort of Irish Wolfhounds both individually, in pairs, and in trios. Some loci were significantly associated with the disease individually, but no genotypes individually or in pairs conferred a significantly greater risk of developing dilated cardiomyopathy than the population risk. Combining three loci together however did result in the identification of a genotype which conferred a greater risk of disease than the overall population risk. This study validates previous work indicating that considering multiple loci together rather than individually could lead to the identification of significant genetic risk factors of disease.

4.2. Introduction

Dilated cardiomyopathy (DCM) is the most common cause of cardiac death in Irish Wolfhounds (IWHs) (Egenvall et al., 2006, Vollmar, 2000). In human DCM there is often a heritable genetic cause of this disease (Mestroni et al., 1999, Posafalvi et al., 2012). In several breeds, including IWHs, canine DCM has been shown to be heritable (Dambach et al., 1999, Distl et al., 2007, Petric et al., 2002). The heritability of canine DCM and its clinical similarity to human DCM suggest that there is also a genetic basis to canine DCM (Mahon, 2005, Mausberg et al., 2011).

The Irish Wolfhound (IWH) is a rare giant dog breed and it is considered to be vulnerable by the UK kennel club due to the small number of puppy registrations each year (Lewis et al., 2015). IWHs are not a long lived breed. Median age at death in the UK population is reported to be 7.04 years, with a maximum reported age of 11.83 years (Adams et al., 2010). In the same survey 165 other breeds were included and only 16 had a lower median age at death than the IWH breed. There were 71 breeds with a median age at death higher than the IWH maximum reported age at death (Adams et al., 2010). There are a number of health problems to which the IWH breed is susceptible that contribute to the low median age at death. These include heart disease, osteosarcoma, pneumonia, and gastric dilation-volvulus (Egenvall et al., 2006, Egenvall et al., 2007, Evans and Adams, 2010, Greenwell and Brain, 2014). As discussed elsewhere in this thesis, canine osteosarcoma ultimately results in death, with survival following diagnosis typically less than one year (Bhandal and Boston, 2011, Egenvall et al., 2007, Mitchell et al., 2016). If diagnosed and treated early, IWHs with heart disease have a better prognosis than those diagnosed with osteosarcoma, with median time to death reported to be up to 4 years following early heart disease diagnosis (Vollmar and Fox, 2016). There is limited literature on pneumonia in IWHs, but Greenwell and Brain (2014) reported a breed predisposition compared to other breeds treated for pneumonia within a specialist small animal hospital. The majority (88.9%) of these initial cases were successfully treated, although 44% were ultimately euthanised due to recurrent pneumonia (Greenwell and Brain, 2014). Although treatable and many individuals survive, gastric dilation-volvulus can be life threatening, with reported death rates of between 20.5% and 30% across all breeds (Adamik et al., 2009, Glickman et al., 2000). Osteosarcoma and heart disease are the most likely causes of death of IWHs, but increased incidence of pneumonia and gastric dilation-volvulus relative to other breeds also lower the median lifespan of IWHs (Egenvall et al., 2006, Egenvall et al., 2007, Evans and Adams, 2010, Greenwell and Brain, 2014). Cardiac disease is the most common specific cause of death in IWHs, thus improving the prevention, diagnosis, and treatment of cardiac disease is likely to have the greatest impact on improving longevity and welfare in the breed (Bonnett et al., 2005).

There have been several studies investigating the genetic basis of canine DCM, but despite this very few genetic associations have been made (Philipp et al., 2012, Mausberg et al., 2011, Meurs et al., 2012, Meurs et al., 2013, Werner et al., 2008). Possible reasons for the lack of associations identified in many studies include the use of inappropriate control populations, incomplete clinical characterisation of control and affected populations, inadequate samples sizes, or an assumption of simple Mendelian inheritance despite the evidence suggesting more complex multi-factorial influences on heritability (Distl et al., 2007). Inappropriate controls have included unaffected individuals from breeds different to the affected individuals, or young individuals that still have the potential to develop disease (O'Sullivan et al., 2011, Philipp et al., 2008a, Wiersma et al., 2008). Many canine DCM studies have also only had small sample sizes of between 5 and 40 individuals, which is unlikely to be large enough to detect a genetic association (Lindblad-Toh et al., 2005). Previous work has shown that multiple loci could be working synergistically to influence canine DCM development, thus loci should be examined together as well as individually for associations with disease (Chapter 3 (Simpson et al., 2015a)). This current study makes use of the largest number of affected individuals combined with associated appropriate older unaffected controls from the same breed and examines multiple loci for an association with canine DCM together as well as individually.

In addition to commonly being diagnosed with DCM, IWHs are frequently diagnosed with atrial fibrillation (AF) (Brownlie and Cobb, 1999, Vollmar, 2000). Despite the presence of AF in a large percentage of dogs with DCM, the mechanistic and clinical relationship between DCM and AF has not been clarified (Brownlie and Cobb, 1999, Martin et al., 2009, Tidholm and Jonsson, 1997, Vollmar, 2000). Although AF is not associated with the development of DCM in people, the presence of AF in people with DCM and heart failure has been shown to negatively influence survival (Unverferth et al., 1984, Wasywich et al., 2010). IWHs can develop DCM without AF, though it seems that <2% of IWHs with AF do not go on to develop DCM (Brownlie and Cobb, 1999, Vollmar, 2000). If AF is a potential precursor to DCM, the time from diagnosis of AF to DCM is important, if it is several years than the presence of AF could be less of

a concern than if it is merely a few months. Also if AF is a precursor to DCM there is the potential to give individuals diagnosed with AF drugs such as the phosphodiesterase III/calcium sensitizing drug, pimobendan, to improve survival (Vollmar and Fox, 2016). In addition to the potential clinical implications of AF diagnosis, if AF can be shown to be related to DCM for genetic association testing both diagnoses can be used to test for an association. This also has implications for individuals included in the unaffected group for genetic association testing, individuals included in the unaffected group must be free of both DCM and AF.

There is some evidence that males are affected by DCM more often, or earlier in life than females (Brownlie and Cobb, 1999, Calvert et al., 1997, Tidholm and Jonsson, 1997, Wess et al., 2010). Previous IWH DCM genetic association studies have either not stated the age of their unaffected IWHs, or they have given no justification for the age that a healthy individual is included in the unaffected control group (Philipp et al., 2007, Philipp et al., 2008a, Philipp et al., 2012). Brownlie and Cobb (1999) found a difference between the sexes in the age of onset of DCM in IWH in 39 individuals. If the age of onset differs between males and females, the age at which an unaffected individual can be included in the unaffected group for genetic association studies should be different for males and females. If males are affected more often than females by DCM or there are differences in the age of onset this raises important questions as to why there might be a difference between the sexes in age or frequency of onset. Therefore this study also aims to confirm whether there is a sex effect and/or age of onset differential in relation to DCM in IWH.

In this study, several hypotheses are tested including the relationship between DCM and AF, sex differences in disease prevalence and age of onset, and the use of multiple genetic loci to establish genetic associations.

4.2.1. DCM and AF relationship

Here the hypothesis that AF is associated with DCM in IWHs is tested by establishing how many individuals diagnosed with DCM were also diagnosed with AF and how long an individual can have a diagnosis of AF prior to being

diagnosed with DCM. Individuals diagnosed with AF, but not DCM were also tested to establish whether they had AF diagnosed for a longer period of time on average than individuals which subsequently developed DCM.

4.2.2. Sex differences in numbers affected and age of disease onset

The hypothesis presented was that male IWHs are diagnosed with both DCM and AF more frequently than females and that the combined incidence of both disorders was higher in males than females. In addition the hypothesis that the age of onset of DCM and AF was different in males as compared to females was tested. Therefore, the age at which an individual can be considered unaffected was established for males and females.

4.2.3. Genetic associations

Previous work showed that multiple loci could act together to cause DCM, thus examining loci individually may not yield significant results (Chapter 3 (Simpson et al., 2015a)). In cases where a locus is associated with DCM, the result may not have clinical implications, for example a locus may be associated with disease, but only explain a small number of cases. However, the examination of multiple loci in combination could improve the reliability and clinical value of genetic associations. The hypothesis that genotypes at multiple loci more comprehensively predict incidences of DCM and AF than individual loci alone was tested by genotyping multiple loci putatively associated with IWH DCM and testing for associations at loci both individually and when combined.

4.3. Methods

4.3.1. Samples and health updates

This study was approved by the University of Nottingham ethics committee in compliance with the Home Office regulations and the Veterinary Surgeons Act. Informed consent was obtained from all dog owners involved in this research. Buccal swabs from 379 IWHs were taken using Isohelix DNA Buccal Swabs (Cell Projects Ltd. UK). The swab was inserted into the dog's mouth either by the owner, vets or trained members of the research group, and rubbed on the cheek for up to 2 minutes to enable optimum cell collection. Swabs were stored as directed by the manufacturer, at room temperature upon collection followed

by 4°C. Any swabs stored for longer than a month prior to DNA extraction were stored at -20°C. Optimisation trials indicated that appropriate quality and quantity of DNA could be obtained following all storage methods. When the buccal swabs were taken, owners completed a short information sheet for each dog in order to obtain current and past health information, date of birth, pedigree information, sex, and neutering status, a copy of the form is shown in Appendix 3. All information was associated with a unique sample number and stored in a secure excel database.

Follow-up data on the health of DNA-sampled dogs was obtained at numerous time points. Owners were asked to fill in a 'health update' form annually or following any change in health/upon death. Forms could be completed either online or in paper format to ensure maximum owner participation, a copy of this form is in Appendix 3. In addition, telephone surveys were carried out in collaboration with undergraduate research project students who were supervised by the PIs on this longitudinal health study. The surveys were utilised to obtain health updates and epidemiology data on all dogs from which samples had been obtained. A copy of the questionnaire is shown in Appendix 4. Many IWHs are presented to specialist veterinary cardiologists on a regular basis for heart disease screening. These heart screenings consist of cardiac auscultation, a six lead electrocardiogram, and complete echocardiographic assessment. Data from these heart screenings were provided by veterinary cardiology specialist Dr Serena Brownlie. Cardiac diagnoses were determined by Professor Malcolm Cobb based on the data from heart screenings. Information from owner health up-dates and heart screenings was added to that stored in the excel database and analysed alongside genetic data.

4.3.2. DCM and AF relationship

Individuals diagnosed with DCM, AF, and both DCM and AF in the database were identified, and the numbers in each diagnosis category "DCM", "AF", DCM and AF" were determined. In the subset of individuals with both DCM and AF, the time from AF diagnosis to DCM diagnosis was established based on the dates of the heart screenings at which each were diagnosed. Individuals that were diagnosed with AF, but not DCM had the time with AF determined by the

time between the first heart screening that AF was diagnosed at and the most recent heart screening. A *t*-test was performed using R statistical software (RCoreTeam, 2015) to establish if the time from AF diagnosis to DCM diagnosis was significantly different from the time that individuals had been known to have a diagnosis of AF, but not DCM.

4.3.3. Sex differences in numbers affected and age of disease onset

The number of males and females diagnosed with DCM or AF was established from the database along with the number of males and females not diagnosed with DCM or AF. A χ^2 test was performed to establish whether there were differences in the number of males and females diagnosed with DCM or AF. The age at first diagnosis of DCM or AF was established for all individuals diagnosed with either disorder. A *t*-test was performed using R statistical software (RCoreTeam, 2015) to identify statistical differences between males and females at age of diagnosis. The data was also displayed visually in bar plots generated using R statistical software (RCoreTeam, 2015).

4.3.4. Genetic associations

4.3.4.1. DNA extraction and restriction digest fragment genotyping

To extract DNA from buccal swabs, the end of the swab was first dissociated into small pieces using scissors which were sterilised between swabs using Anistel (Tristel, UK) and then wiped down with Industrial Methylated Spirits (IMS) (Thermo Fisher Scientific, USA). The small pieces of swab were put into a sterile 1.5mL microcentrifuge tube (Eppendorf, Germany). The DNA-containing swab fragments were re-suspended in 600µL of a solution composed of 5% sodium Chelex® 50-100 mesh (dry) (Sigma-Aldrich, UK) reconstituted in 1xTE (10mM Tris (Thermo Fisher Scientific, USA)), 1mM EDTA (Thermo Fisher Scientific, USA) in H_2O, pH 8.0) and 20µL of 10mg/mL proteinase K (Promega, USA), 50mM Tris (Thermo Fisher Scientific, USA), 10mM $CaCl_2$ (Thermo Fisher Scientific, USA) pH 8.0. The sample-containing microcentrifuge tube was vortexed to mix the components. The microcentrifuge tube was then placed in a water bath at 45°C overnight to digest the cells. The sample was then vortexed once more before incubation in a heating block at

100°C for 8 minutes to inactivate the proteinase K. The sample was then centrifuged at 13,500xg for 3 minutes to separate the Chelex® and swab from the supernatant. The DNA-containing supernatant was then transferred into a sterile 1.5mL microcentrifuge tube and was used in subsequent PCRs. The extracted DNA was then stored at -20°C in the Nottingham Comparative Genomic Biobank.

Restriction digests were used to genotype SNPs previously shown to be associated with DCM (Philipp et al., 2012), in the Nottingham IWH cohort. In the first instance the effect of the SNPs on restriction sites within the affected loci were examined. PCR primers were designed to flank five DCM-associated SNPs which alter restriction sites. Where the SNP did not alter or create a restriction site, PCR primers were designed to introduce a restriction site into the PCR product to detect the presence/absence of the SNP. Primer information and fragment size amplified is presented in Table 4.1. Primers were obtained from Sigma-Aldrich, UK. The SNPs are hereby referred to as Chr1 for rs21953123, Chr10 for rs22078677, Chr15 for rs22422063, Chr21 for rs22923291, and Chr37 for rs24025150.

The PCR reaction mixture consisted of the following: 1x LightCycler® 480 Probes Master Mix (Roche, Switzerland), primers at a concentration of 0.5µM, between 2 and 4µL of template DNA per reaction, adjusted to 25µL using PCR grade H_2O. PCR reactions were carried under the following conditions: Initial denaturation 94°C for 30 seconds, 40 cycles of: 94°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 30 seconds, final extension 72°C for 5 minutes. The annealing temperature optimisation was determined by performing a temperature gradient PCR between 52°C and 64°C. Annealing temperatures for each SNP are in Table 4.2.

Restriction digests were conducted on each PCR product. The reaction mixture for each restriction digest consisted of 3µL of 10x Buffer, 20µL PCR product, and 1-6 units of enzyme in a reaction volume of 30µL. The enzyme manufacturer provided the appropriate buffer for each enzyme and the stated activation temperature, the number of units of enzyme was optimised for each

restriction digest reaction. The details for each restriction digest are shown in Table 4.3. The restriction digests were performed by incubating the reaction mixture at the activation temperature of enzyme for 14hours.

Following digestion, the digested PCR products were electrophoresed at 100v for 45 minutes on 2% agarose 1X TAE gels stained with Nancy-520 (Sigma-Aldrich, USA) in 1X TAE buffer. A DNA size ladder (ϕx-HaeIII fragments) purchased from NEB was used to determine the size of the digested PCR fragments. Genotypes were determined by the restriction digestion fragment pattern based on the presence of an appropriate restriction site determined by the presence of the SNP, with the possibility of detecting homozygotes (AA, aa) and heterozygotes (Aa). Presumptive homozygotes for each genotype (AA, aa) were confirmed by direct Sanger sequencing of representative PCR fragments (Source BioSciences, Nottingham). For all loci a single band the same size as the PCR product showed that none of the PCR product had been digested and was genotyped as a homozygote for the allele that does not digest. The absence of this larger band and the presence of smaller bands at the expected sizes following digestion (shown in Table 4.1) showed that all of the PCR product had been digested so these were genotyped as homozygotes for the allele that was digested by the restriction enzyme. Those with a band the same size as the PCR product plus bands at the expected sizes following successful digestion in Table 4.1 were genotyped as heterozygotes. These genotypes were manually recorded within a database alongside appropriate sample numbers and clinical information. One sample of each genotype at each SNP was confirmed by Sanger sequencing by Source Bioscience (Nottingham, UK).

Table 4.1. PCR primer information.

SNP	Primer pair	Forward primer sequence	Reverse primer sequence	PCR fragment length
Chr1: rs21953123 C/T	initial primers	TCCTCTCCAAACATTAGAAAAAGACC	ACTACATATAGTCAGCTTTACAGATTG	311bp
	additional primers	TATCGTACTTTATAATGAAAGATTGAATG	AGAATTTAATATCTGAAAATATATCAACAC	417bp
Chr10: rs22078677 A/C	initial primers	CCTGGTGTCAGAAACACAAGgCAC	TCAAGTAGCTTAAACTCTCAGGGCC	374bp
	initial forward primer, additional reverse primer		TGTGTATTCATCAGCTCTTCCTAGG	161bp
Chr15: rs22422063 C/A	initial primers	AATCATTTGACAGTTAGGTTAACTTCC	CACAATTTGCACATTACACATTATTAAC	375bp
	initial forward primer, additional reverse primer	GCTGGTAAACATTCTTAAATTCAGCG	ATTTGAGTTTTCCCTCACATTATTGAC	443bp
Chr21: rs22923291 G/A	initial primers	GAAACTCCAGTCATGTTTAACTATTTG	GAAGAGCAGTAATGACTAATACgCAG	349bp
	additional forward primer, initial reverse primer	TGTTCATCTCTCACAGGACTTGAG		410bp
Chr37: rs24025150 G/A	initial primers	GGAAACTGGTTTCCAATGAAGAcC	ACGTAACAAGTTTGAAGTGTTGCTG	374bp
	initial forward primer, additional reverse primer		CCTGCCACCTGTGTTACTTTACGC	133bp

DCM associated SNP locations in genome (from Philipp *et al.* (2012)), PCR primer sequence information, if the primer pair is the initial or new primers, and resulting PCR product sizes. Primer sequence in lower case indicates a base change from the reference sequence to create a restriction digest site.

Table 4.2. PCR annealing temperatures as determined by temperature gradient PCR optimisation for each primer pair.

SNP	Primer pair	Annealing temperature
Chr1: rs21953123 C/T	all primer pairs	52°C
Chr10: rs22078677 A/C	initial primers	60°C
	initial forward primer, new reverse primer	52°C
Chr15: rs22422063 C/A	all primer pairs	52°C
Chr21: rs22923291 G/A	initial primers	58°C
	new forward primer, initial reverse primer	52°C
Chr37: rs24025150 G/A	initial primers	58°C
	initial forward primer, new reverse primer	52°C

Table 4.3. Restriction digest information.

SNP	Enzyme	units of enzyme per reaction	Restriction site	cuts allele	Primer pair	Digest fragment sizes
Chr1: rs21953123 C/T	HpyCH4II (NEB, USA)	3	ACTGT	C	original primers	228 & 83
					new primers	260 & 157
Chr10: rs22078677 A/C	BanI (NEB, USA)	6	GgCACC	C	original primers	19 & 355
					original forward primer, new reverse primer	19 & 142
Chr15: rs22422063 C/A	NciI (NEB, USA)	6	CCCGG	C	original primers	196 & 179
					original forward primer, new reverse primer	227 & 216
Chr21: rs22923291 G/A	Fnu4HI (NEB, USA)	1	GCTGc	G	original primers	310 & 39
					new forward primer, original reverse primer	384 & 39
Chr37: rs24025150 G/A	HpaII (NEB, USA)	3	cCGG	G	original primers	28 & 346
					original forward primer, new reverse primer	28 & 105

Enzymes, restriction sites, primer pairs and digest fragment sizes for each SNP.

4.3.4.2. Analysis

To establish whether the population within this study is genetically consistent with the data published by Philipp *et al.* (2012), the allele frequencies at each locus were determined in the entire sampled population irrespective of age or disease status. These allele frequencies were compared to the published allele frequencies at the same loci.

All individuals with a diagnosis of DCM or AF were included in the affected group. As outlined later males were found to be affected by DCM and AF at an age earlier than females. For this reason, females were considered to be unaffected if they had not been diagnosed with either DCM or AF at a heart test after the age of 8.5 years. Males were considered unaffected if they had reached the age of 6.5 years and had a heart test without a diagnosis of DCM or AF.

The number of individuals expected to develop DCM under four common penetrance models for each SNP was determined by methods described by Camp (1997). The observed risk of disease for each genotype was obtained directly from the data by dividing the number of each genotype affected by the total number of individuals with the genotype. The genetic penetrance parameter (γ) was then established for each SNP. For the multiplicative, additive, and dominant models, γ indicates the relative risk of the heterozygote genotype compared to the homozygote genotype with the lowest risk, for the recessive model it indicates the relative risk of the most at risk homozygote compared to the least at risk homozygote. The penetrance parameter was then used to calculate the proportion of each genotype expected to develop disease under each model. Table 4.4 shows how the penetrance parameter was used with the lowest homozygote risk under each model to calculate these proportions.

Table 4.4. The relationship of the risk of disease with the penetrance parameter.

	Penetrance		
Model	**a/a**	**a/A**	**A/A**
Multiplicative	f_0	$f_0\gamma$	$f_0\gamma^2$
Additive	f_0	$f_0\gamma$	$2f_0\gamma$
Recessive	f_0	f_0	$f_0\gamma$
Dominant	f_0	$f_0\gamma$	$f_0\gamma$

The risk of disease (f_0) of the least risky homozygote (a/a) is related to the genetic penetrance parameter (γ) for each additional genotype under multiplicative, additive, recessive, and dominant models. The penetrance parameter is a measure of the additional risk of disease that the risky allele confers to an individual. For the multiplicative, additive and dominant models γ is the relative risk of the heterozygote genotype compared to the homozygote genotype with the lowest risk, for the recessive model it is the relative risk of the most at risk homozygote compared to the least at risk homozygote.

The proportion of each genotype expected to develop disease under each model was then translated into the number expected by multiplying the proportion by the total number of individuals observed with the genotype, irrespective of disease status. A χ^2 test was performed to establish which model of penetrance most closely fitted the observed data. The model with the smallest χ^2 value was taken to be the best fit of the observed data.

Having established the model of penetrance, the most appropriate test for association with disease was then carried out. Where A is the disease associated allele, for dominant models the counts for individuals which genotyped as a/A and A/A can be pooled as the penetrance is equivalent, similarly for recessive models the counts for individuals genotyped as a/a and a/A can be pooled (Clarke et al., 2011). For multiplicative models allelic tests of association are more powerful (Clarke et al., 2011). Additive models are most appropriately tested by a Cochran-Armitage trend test, or genotype association testing (Clarke et al., 2011). To test for association with disease χ^2 tests were performed. The expected numbers of affected individuals with each genotype or allele were obtained by establishing the proportion of unaffected individuals with each genotype or allele. These proportions of unaffected individuals with each genotype or allele were then multiplied by the total number of affected

individuals to establish the expected number of affected individuals with each genotype. To account for multiple tests a Bonferroni correction was applied to the p values.

To establish whether particular genotypes gave an increased risk compared to the overall population risk, relative risks were calculated alongside 95% confidence intervals. Relative risks were calculated by dividing the genotype risk with the population risk and 95% confidence intervals were calculated using the methods in (Morris and Gardner, 1988). Where appropriate, genotypes were combined, for example where the penetrance model was shown to be recessive or dominant, or where there were less than 5 total individuals with a genotype.

To establish whether using multiple loci to test for an association could increase the association compared to individual loci alone, each significant locus was considered with each of the other significant loci in pairs and all combined. For this analysis the expected numbers were calculated and tested for an association in the same way as for loci individually. Risks of disease for each genotype combination were also established and relative risks calculated in the same way as individual loci.

4.4. Results

4.4.1. DCM and AF relationship

There were 36 individuals diagnosed with DCM in the current study (from a total of 379 genotyped individuals – 9.50%), 29 (80.5%) DCM affected individuals also had a diagnosis of AF. In total, 17 of the 29 (58.6%) were diagnosed with DCM and AF at the same time, while the remaining 12 had been diagnosed with AF prior to the DCM diagnosis. Of the 12 that had been diagnosed with AF prior to DCM, 11 (91.7%) had been diagnosed with AF within 2 years, and all had DCM diagnosed within 3 years of their first AF diagnosis.

In the current study, there were also 36 individuals (from a total of 379 genotyped individuals – 9.50%) that had been diagnosed with AF, but not DCM. Of these individuals 24 (66.7%) had not been presented for heart disease

screening since diagnosis. Only 7 (19.4%) had only been tested within 1 year of their original diagnosis of AF. A further 4 individuals (10.8%) had been tested within 2 years of original diagnosis and one individual had been tested 2.5 years following AF diagnosis.

The time from AF diagnosis to DCM diagnosis in the group of individuals diagnosed with both AF and DCM was not significantly different from the time individuals only diagnosed with AF had been known to have a diagnosis of AF for (t = 0.56, p = 0.58).

4.4.2. Sex differences in numbers affected and age of disease onset

Individuals with diagnoses of DCM and AF were included in the affected group. There was no difference in the proportion of males and females affected by DCM/AF combined – χ^2 test result = 0.13 (p = 0.72, df = 1). There was a significant difference between males and females in the age of diagnosis of DCM/AF combined. DCM/AF combined mean age at diagnosis was 4.82 years for males and 6.14 years for females (t = 2.43, p = 0.019). Of males that developed AF or DCM, 80% had done so before the age of 6.5 years, while 80% females that developed AF or DCM, did so before the age of 8.5 years (Figure 4.1). Using these age restrictions in the sample cohort there were 9 females unaffected by either diagnosis over the age of 8.5 years old and 14 males unaffected over 6.5 years old, see

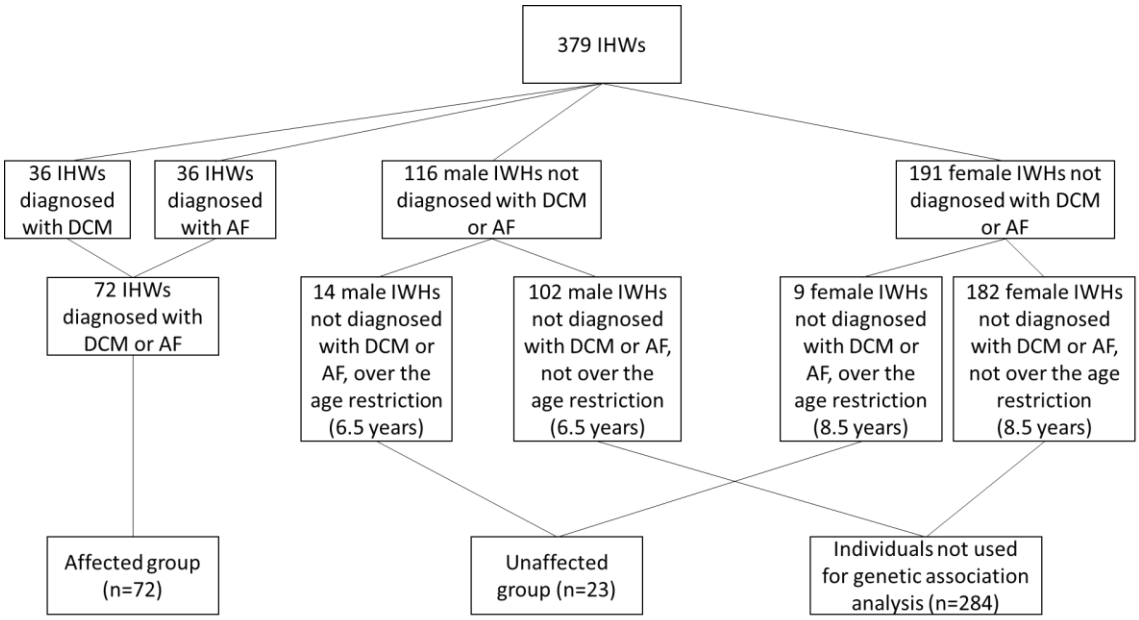


Figure **4.2**. Of the individuals not included in the DCM/AF affected group or the unaffected group, 20 were known to have died of causes other than DCM or AF before reaching the age restrictions, 234 had not reached the age restrictions, and 27 were over the age restriction but had not been heart tested since reaching the age restriction, and 3 were of unknown age. Using the information above, it was therefore possible to define the 'unaffected' group with a high level of stringency and specificity, in addition to enhancing the knowledge on sex related differences in age of clinical diagnosis.

It was noted that there was no difference in observed and expected numbers of neutered affected cases in either males or females (n=191 entire females and 66 neutered females, with 28 and 16 affected respectively. n=116 entire males and 25 neutered males with 16 and 7 affected respectively. $\chi^2 = 2.63$, $p>0.05$ for females and $\chi^2 = 2.54$, $p>0.05$ for males)

Within the study cohort, 23 (34.7%) individuals were diagnosed with DCM or AF at their first heart test and of these, 19 (82.6%) were less than 6.5 years old. Of the 4 that were over 6.5 years old at first diagnosis without a previous heart test, 1 was male and 3 were female. Due to the uncertainty in the age that these older individuals would have developed the condition, these individuals were removed from the age of onset analysis to ensure that these do not skew the difference in age of onset between the sexes. The *t*-test result excluding these individuals remained significant at the 5% threshold level ($t = 2.11$, $p = 0.040$), which was comparable to when those individuals were included within the dataset.

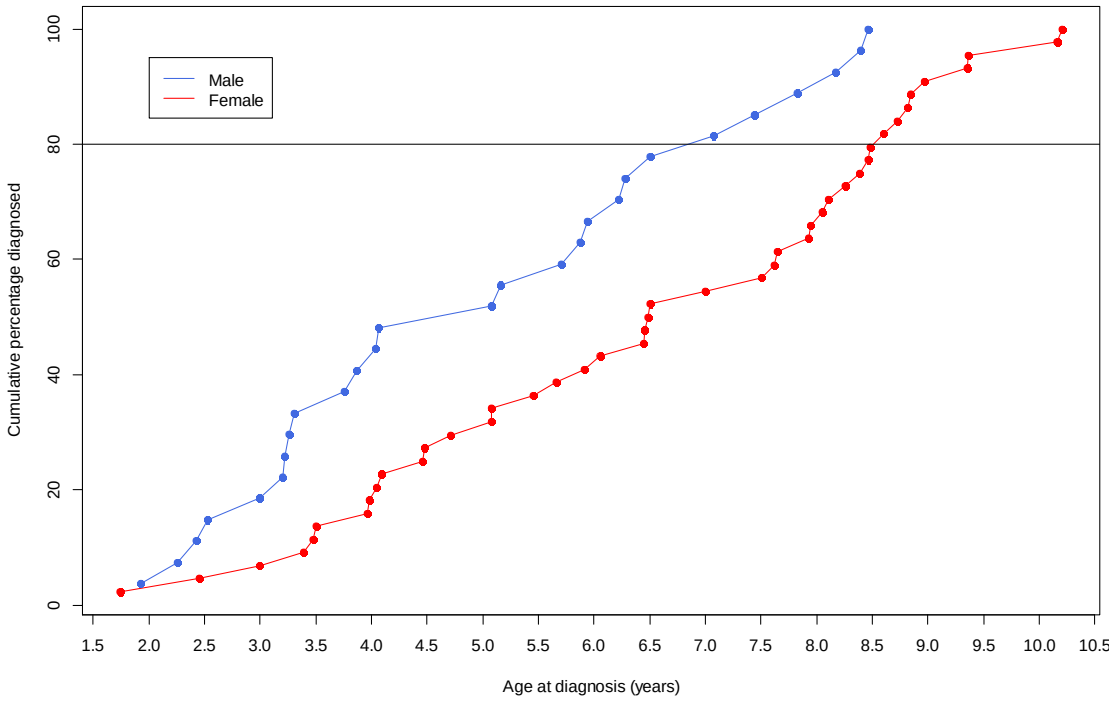


Figure 4.1. Cumulative percentage of individuals diagnosed with DCM or AF at or before each age category. Males (blue) and females (red) are shown separately.

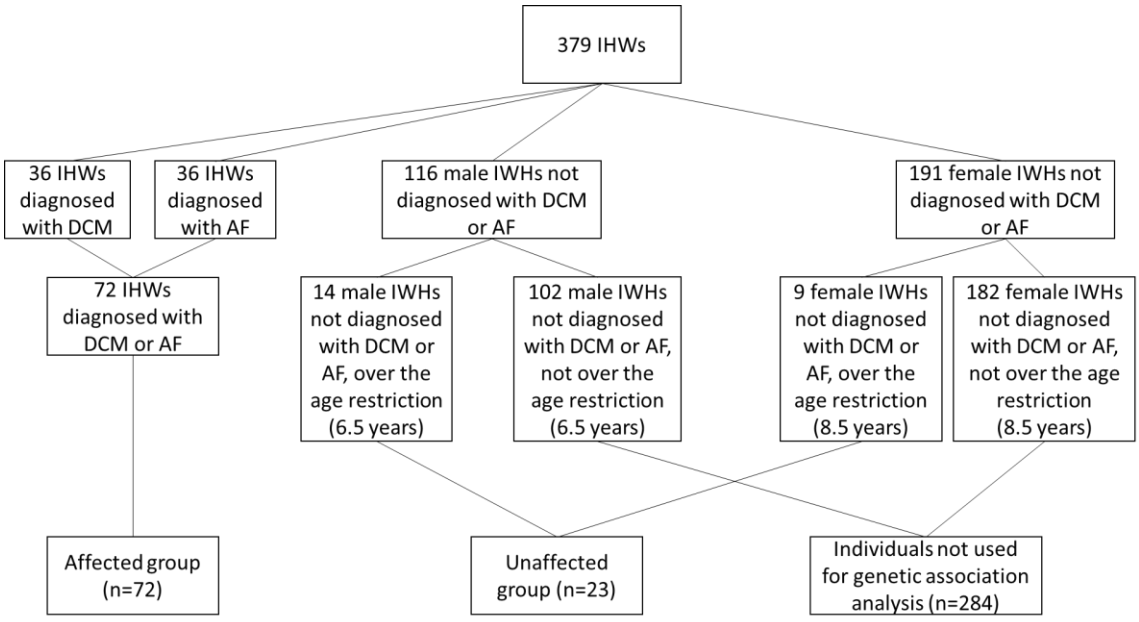


Figure 4.2. Diagram detailing which group individuals were included in for DCM/AF genetic association analyses. Final groups included "Affected", "Unaffected" and "not used for genetic association analysis"

4.4.3. Genetic associations

The individuals in the current study were primarily from the UK IWH population, while those in the study by Philipp *et al.* (2012) were from mainland Europe. Despite the use of a different sub-population of IWHs there was generally a concordance between the published allele frequencies and those in the current study (Table 4.5). The alleles at the SNPs on chromosomes 1, 10, and 37 were within 0.1 of the published frequencies. The alleles at the SNPs on chromosome 15 and 21 were close to within 0.1 of the published frequencies.

Table 4.5. Allele frequencies at each SNP in the data published in Philipp *et al.* (2012) and the current study.

Allele	published allele frequency	current study allele frequency
Chr1 C	0.88	0.82
Chr1 T	0.12	0.18
Chr10 A	0.94	0.94
Chr10 C	0.06	0.06
Chr15 C	0.74	0.63
Chr15 A	0.26	0.37
Chr21 G	0.71	0.58
Chr21 A	0.29	0.42
Chr37 G	0.57	0.59
Chr37 A	0.43	0.41

Chr1 refers to the SNP on Chromosome 1, Chr10 the SNP on Chromosome 10, etc. as identified in Philipp *et al.* (2012) and Table 4.1.

The mode of penetrance was established to be multigeneic for the SNPs on chromosomes 1 and 10, dominant for the chromosome 15 and 21 SNPs, and recessive for the SNP on chromosome 37 (Table 4.6). This allowed allelic association tests to be performed for the SNPs on chromosomes 1 and 10, the numbers of individuals genotyped as AA and AC for SNP on chromosome 15 to be combined, and the numbers of individuals genotyped as GA and GG for the SNPs on chromosomes 21 and 37 to be combined, thus the number of degrees of freedom for each association test was reduced to one (Table 4.6).

Table 4.6. Mode of penetrance for individual SNPs in relation to IWH DCM/AF.

	chr1	chr10	chr15	chr21	chr37
mode of penetrance	multigenic	multigenic	A dominant	G dominant	A recessive
type of test	allelic	allelic	A dominant	G dominant	A recessive
chi-squared test result	10.61	5.07	1.52	7.67	11.12
df	1	1	1	1	1
Bonferroni corrected p	0.0056**	0.12	1.00	0.028*	0.0043***
Published DCM allele	C	A	C	A	G
Current DCM/AF allele	C	C	A	G	A

Showing the mode of penetrance, subsequent genetic association test used, chi-squared test result, degrees of freedom of the chi-squared test, and Bonferroni corrected p value for each locus. Also shown are the alleles associated with IWH DCM in Philipp *et al.* (2012) and IWH DCM/AF in the current study. Chr1 refers to the SNP on Chromosome 1, Chr10 the SNP on Chromosome 10, etc. as identified in Philipp et al. (2012) and Table 4.1. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$

Three loci had significant associations with DCM/AF combined, specifically the SNPs on chromosome 1, 21, and 37 (Table 4.6). The risks of developing DCM or AF for each genotype are presented in Table 4.7, along with the total numbers of individuals with each genotype. There were very few individuals genotyped as TT at the chromosome 1 locus, CC was the most risky allele and allelic association tests established that the C allele was associated with DCM/AF, therefore the numbers of TT individuals were combined with the CT individuals. The Chromosome 21 and 37 SNPs were determined to be dominant and recessive respectively so the GA and GG genotypes were combined at both loci. The overall risk of developing DCM or AF was 0.76. Several genotypes gave an elevated risk compared to the overall risk, and several gave reduced risk, none of these increased or decreased risks were significantly different from the overall risk.

Combining genotypes at pairs of loci resulted in smaller p values for two out of three combinations than when loci were considered individually (Tables 4.6 and 4.8). Combining all three loci together further reduced the p value for all loci (χ^2 value = 41.09, p = 0.00000078).

From both the pairs of loci and all three loci combined, the genotypes with the greatest disease risk were those which combined the high risk individual genotypes (Tables 4.8 - 4.12). In particular Chr1 CC, Chr21 GA/AA, and Chr37 AA combined gave the single highest risk of developing disease when more than four individuals were genotyped, and was significantly higher than the overall population risk. The opposite was also the case with the lowest combined disease risk resulting from the combined genotypes of those with individually the lowest risk, but none gave a significantly decreased risk compared to the overall population risk.

Table 4.7. Risk of developing DCM/AF for each genotype at loci identified as significantly associated with DCM/AF.

Genotype	risk	Total genotyped	Risk relative to population risk	95% CI
Chr1 CC	0.81	54	1.08	0.91 to 1.28
Chr1 CT	0.68	22	na	na
Chr1 TT	0.5	4	na	na
Chr1 CT/TT	0.65	26	0.86	0.64 to 1.17
Chr21 AA	0.61	18	0.81	0.55 to 1.19
Chr21 GA	0.77	39	na	na
Chr21 GG	0.82	33	na	na
Chr21 GA/GG	0.79	72	1.04	0.89 to 1.23
Chr37 AA	0.85	27	1.12	0.93 to 1.36
Chr37 GA	0.70	23	na	na
Chr37 GG	0.73	44	na	na
Chr37 GA/GG	0.72	67	0.95	0.78 to 1.14

The risk of developing disease for each genotype, the total number of individuals with each genotype, the relative risk of each genotype compared to the population risk, and associated 95% confidence intervals. Where dominant or recessive modes of penetrance were established, or less than 5 total individuals had a genotype, genotypes were pooled and these are also presented. Chr1 refers to the SNP on Chromosome 1, Chr21 the SNP on Chromosome 21, and Chr37 the SNP on Chromosome 37 as identified in Philipp *et al.* (2012) and Table 4.1.

Table 4.8. Chi squared test results for the genotype associations with DCM/AF for pairs of loci.

	chr1+21	chr1+37	chr21+37
chi-squared test result	14.16	15.56	24.55
df	3	3	3
Bonferroni corrected p	0.0081**	0.0042***	0.000058***

Includes chi-squared test results, degrees of freedom (df) of the chi-squared tests, and Bonferroni corrected p values. Chr1 refers to the SNP on Chromosome 1, Chr21 the SNP on Chromosome 21, and Chr37 the SNP on Chromosome 37 as identified in Philipp *et al.* (2012) and Table 4.1. ** = p<0.01, *** = p<0.005

Table 4.9. The risk of developing disease for each genotype combination at the chromosome 1 and 21 loci.

chr1	chr21	DCM/AF risk	Total genotyped	Risk relative to population risk	95% CI
CC	**GG/GA**	0.81	48	1.07	0.90 to 1.28
CC	AA	0.83	6	1.10	0.76 to 1.60
CT/TT	**GG/GA**	0.74	19	0.97	0.73 to 1.30
CT/TT	AA	0.33	6	0.44	0.14 to 1.37

Showing the total number of individuals with each genotype combination, the relative risk of each genotype compared to the population risk, and associated 95% confidence intervals. The individual genotypes are in bold type if they were identified as conferring higher risk of disease and normal type if they were identified as conferring lover risk of disease.

Table 4.10. The risk of developing disease for each genotype combination at the chromosome 1 and 37 loci.

chr1	chr37	DCM/AF risk	Total genotyped	Risk relative to population risk	95% CI
CC	GG/GA	0.79	38	1.04	0.85 to 1.27
CC	**AA**	0.88	16	1.15	0.93 to 1.43
CT/TT	GG/GA	0.61	18	0.81	0.55 to 1.19
CT/TT	**AA**	0.75	8	0.99	0.65 to 1.50

Showing the total number of individuals with each genotype combination, the relative risk of each genotype compared to the population risk, and associated 95% confidence intervals. The individual genotypes are in bold type if they were identified as conferring higher risk of disease and normal type if they were identified as conferring lower risk of disease.

Table 4.11. The risk of developing disease for each genotype combination at the chromosome 21 and 37 loci.

chr21	chr37	DCM/AF risk	Total genotyped	Risk relative to population risk	95% CI
AA	GG/GA	0.62	13	0.81	0.52 to 1.27
AA	**AA**	0.60	5	0.79	0.38 to 1.63
GA/GG	GG/GA	0.75	52	0.99	0.82 to 1.20
GA/GG	**AA**	0.89	19	1.18	0.97 to 1.43

Showing the total number of individuals with each genotype combination, the relative risk of each genotype compared to the population risk, and associated 95% confidence intervals. The individual genotypes are in bold type if they were identified as conferring higher risk of disease and normal type if they were identified as conferring lower risk of disease.

Table 4.12. The risk of developing disease for each genotype combination at the chromosome1, 21, and 37 loci.

chr1	chr21	chr37	DCM/AF risk	Total genotyped	Risk relative to population risk	95% CI
TT/CT	AA	**AA**	0.50	2	0.66	0.16 to 2.65
TT/CT	AA	GG/GA	0.25	4	0.33	0.06 to 1.81
TT/CT	**GA/GG**	**AA**	0.80	5	1.06	0.67 to 1.66
TT/CT	**GA/GG**	GG/GA	0.73	15	0.97	0.70 to 1.34
CC	AA	**AA**	0.50	2	0.66	0.16 to 2.65
CC	AA	GG/GA	1.00	4	1.32	1.18 to 1.48
CC	**GA/GG**	**AA**	0.93	14	1.23	1.02 to 1.47
CC	**GA/GG**	GG/GA	0.76	34	1.01	0.81 to 1.26

Showing the total number of individuals with each genotype combination, the relative risk of each genotype compared to the population risk, and associated 95% confidence intervals. The individual genotypes are in bold type if they were identified as conferring higher risk of disease and normal type if they were identified as conferring lower risk of disease.

4.5. Discussion

The data acquired here supports an association between AF and DCM in the IWHs included in this study. The majority (80.5%) of individuals with DCM also had a diagnosis of AF in IWHs. Most individuals were diagnosed with AF at the same time as DCM or in the 2 years prior to the diagnosis of DCM, which indicates that AF may be a precursor to a clinical diagnosis of DCM. The current results are consistent with a previous study which found that 87.6% of

individuals diagnosed with DCM were also diagnosed with AF (Vollmar, 2000). Further evidence for the relationship between AF and DCM comes from the duration between initial AF diagnosis and either subsequent heart testing or DCM diagnosis. The majority (66.7%) had not had a subsequent heart test and of those that had been re-tested the longest duration between initial AF diagnosis and most recent heart test was 2.5 years. All individuals with AF alone still have the potential to develop DCM as the time periods with AF were not longer than the time periods between AF diagnosis and DCM diagnosis (t = 0.56, p = 0.58). This finding is consistent with Vollmar (2000) in which 11 individuals were diagnosed with AF, but not DCM, however only 3 individuals were known not to have subsequently developed DCM, with 29 months as the maximum time with AF alone. This relationship between AF and DCM allowed for the inclusion of individuals diagnosed with AF as well as DCM in the affected group for genetic association tests. In addition, this provides evidence that individuals diagnosed with AF should be carefully monitored and regularly presented for heart testing to ensure that they do not yet require treatment for DCM. There is also the potential to improve the survival of individuals diagnosed with AF by treating them with drugs such as pimobendan prior to the development of DCM or heart failure (Vollmar and Fox, 2016).

Very few IWHs were unaffected by AF or DCM into old age (defined as older than the median lifespan of 7.04 years in IWHs (Adams et al., 2010)) as most individuals developed one or both disorders, or they died of other conditions before reaching old age. As noted earlier the breed is also susceptible to developing osteosarcoma, pneumonia, and gastric dilation-volvulus, all of which can result in death (Egenvall et al., 2007, Evans and Adams, 2010, Greenwell and Brain, 2014). Due to the lack of unaffected older individuals, it was necessary to arbitrarily determine at what age to allow an individual to be included in the unaffected by DCM or AF group for genetic association testing. Young unaffected individuals had a lot of potential to develop DCM or AF in the future, thus including these individuals in the unaffected group would have resulted in misleading genetic association test results. Ideally only very old unaffected individuals would have been included in the unaffected group as the certainty that they have not developed DCM or AF would have been high.

Although this would have reduced the risk of including an individual in the wrong group, it would have reduced the size of the unaffected group to an unpractical size for genetic association testing. As a compromise the age at which 80% of ultimately affected individuals were affected by was determined and this age restriction utilised to determine at what age to allow an unaffected individual to be included in the unaffected group. In the present study, the age of disease diagnosis was established as significantly different between males and females. Males were diagnosed with DCM/AF on average younger than females, although individuals of both sexes were diagnosed from the age of 1.5 years (Figure 4.1), this corroborates the findings of Brownlie and Cobb (1999). The age at which 80% of all ultimately AF/DCM affected males was established as 6.5 years. Females, however, needed to reach 8.5 years before 80% of the AF or DCM affected individuals were diagnosed. These age restrictions were used to determine at what age it was practical to assign a currently unaffected individual to the 'unaffected control' group. Despite the collection of 379 samples across four years, and the use of lenient age restrictions, there were only 22 individuals not affected by DCM or AF above these two ages. Some of the individuals that were not included in either group had died before reaching the age restrictions, but the majority (261 individuals) had either not reached the age restriction by the end of the study, or had not been heart tested since reaching the age restriction. There was a clear difference in the age of diagnosis between males and females, but the reasons for this are currently unknown. It was possible that males were heart tested earlier in life and more regularly than females thus resulting in earlier diagnosis of males than female. The calculations which involved exclusion of individuals diagnosed over the age of 6.5 years of both sexes which had not previously been heart tested did not change the significance of the *t*-test determining differences in the age of diagnosis. This indicated that the differences in the age of diagnosis are likely to be related to the age of disease onset, not age at presentation for heart testing. Strong candidates for further work to investigate these sex differences are hormones which differ in the amount and ratio between the sexes, and the different responses to these hormones (Dabbs, 1990, Kushnir et al., 2010, Lagergren et al., 2015, Morales et al., 1998, Rosenfield and Otto, 1972) (Chapter 6). In light of the differences between males and females, neutering

status was of interest. There was not an overall difference in the number of affected cases between neutered and non-neutered IWHs for either males or females, however further investigations into neutering age could be investigated to show whether this affects disease onset or progression.

All loci examined for an association with disease had previously been associated or putatively associated with DCM in IWHs from mainland Europe by Philipp *et al.* (2012). The current study primarily studied IWHs from the UK and genetic differences between sub-populations of the breed were possible. Whilst this potential genetic difference was possible, it was unlikely as mixing of bloodlines occurs throughout Europe (IWDB, 2016). This was supported by the finding that allele frequencies were broadly similar in both genotyped populations for all loci (Table 4.5). Given the genetic similarity between the mainland Europe IWH population and the UK IWH population, genetic associations with disease in one population should be true in the other, but this was not the case (Table 4.6). Only three out of five of the SNPs previously associated with IWH DCM in mainland European dogs were associated with DCM/AF in the current study, and of these only one had the same allele associated with disease (Table 4.6) (Philipp et al., 2012). There are a number of possible explanations for this inconsistency between studies. The first is that despite genetic admixture occurring and the similarity in allele frequencies between populations, there was unidentified population substructure. Although this is possible it is unlikely given the known mixing of bloodlines and the meticulous record keeping required for prestigious pedigree breeds such as IWHs (IWDB, 2016). More likely explanations for the discrepancy between populations are the sample size and age at which unaffected individuals were included in the unaffected group. Although our study obtained the largest number of samples in the breed ever collected thus far (n = 379), and the previous study by Philipp *et al.* (2012) was large (n = 190) in comparison to many canine genetic studies, for accurate genotype-phenotype associations to be made a larger number of samples is required. Typical human genotype association studies use over 2,000 individuals, with some using more than 10,000 individuals (Cleynen et al., 2016, Hunter et al., 2007, Litchfield et al., 2015, Scott et al., 2007). Philipp *et al.* (2012) used a total of 190 individuals,

and in the present study 379 DNA samples were collected but following more stringent inclusion criteria only 95 individuals could be included in the genetic association analysis. The age an individual can be considered healthy for DCM genetic association purposes varies between breeds, but it has not previously been based on clear justification (Philipp et al., 2007, Philipp et al., 2008a, Philipp et al., 2012). The analysis in the present study shows the age that an unaffected male can be included in the healthy group was younger than an unaffected female (Figure 4.1). The age that an unaffected female can be included in the unaffected group was 8.5 years, the caveat is that despite this age restriction, 20% of females that developed DCM or AF did so after this age. The study by Philipp *et al.* (2012) included unaffected individuals of both sexes over the age of 7 years in the healthy group. In the context of the present results this was adequate for males, for which 80% of affected individuals were diagnosed before the age of 6.5 years. In females, however, over 40% of individuals that developed disease in the current study did so after 7 years old (Figure 4.1), thus the inclusion of females under the age of 8.5 years in the unaffected group is likely to lead to spurious genetic associations in the study by Philipp et al (2012). In addition Philipp *et al.* (2012) did not state whether individuals with AF were excluded from the unaffected group. Although the current study only had 23 individuals in the unaffected group, the genetic associations made are more likely to be correct as individuals were less likely to be inappropriately included in the unaffected group based on age or diagnosis of AF.

Individually, three loci were significantly associated with DCM/AF in the current study, but none of the genotypes conferred a significant increase or decrease in risk compared to the overall population risk of disease (Tables 4.6 and 4.7). Combining the significant loci into pairs increased the significance of the genetic associations, and the risk of disease for the most risky genotype combinations (Tables 4.8 - 4.11). Despite combining genotypes at pairs of loci, none of genotype risks were significantly increased or decreased relative to the population risk. The combination of genotypes from all three significant loci resulted in a significant genetic association ($p = 0.00000078$). Furthermore, the risk of disease in individuals which had the most risky genotypes at all three

loci (Chr1 CC, Chr21 GA/GG, Chr37 AA) was significantly increased compared to the population risk (Table 4.12). This is consistent with our previous work which showed that information from multiple loci combined could be more informative in predicting disease than individual loci and is the first study to show that the multiple allele effect is important in canine heart disease (Simpson et al., 2015a) (Chapter 3).

Genetic associations identify loci and alleles associated with disease, but to be of relevance to clinicians, breeders, and owners, there is a need to define genotype combinations which significantly increase or decrease the risk of developing disease. Individuals with high risk genotypes can be more intensively monitored for the early signs of disease. With heart testing it is possible to detect disease in advance of overt clinical symptoms and it has been shown that some drugs can increase the time to progression to overt disease, thus improving quality of life and longevity (Vollmar and Fox, 2016). If high risk genotype combinations can be identified, breeders can reduce the risk of individuals developing disease by ensuring that matings will not result in offspring with genotypes with a high risk of developing disease. The methodology in this study has the potential to supplement those currently utilised in establishing genetic risk scores, as it can account for interactions between loci and dominant or recessive alleles (Belsky et al., 2013, Ibrahim-Verbaas et al., 2014, Ripatti et al., 2010, Ye et al., 2016). The methodology could be applied to a variety of diseases and traits, and it is not restricted to use in canine disease so it could thus be of use in human genetic counselling.

There were no genotypes identified which conferred a significant protective effect, but some genotypes had few (<5) individuals genotyped irrespective of disease status (Tables 4.7 - 4.12). From so few genotyped individuals it is not possible to draw conclusions, but within some of these genotypes there were more individuals without disease than with disease. This suggests that these genotypes may confer a protective effect against the development of DCM/AF compared to the overall population risk, a greater number of affected and unaffected individuals would allow this to be tested. The inclusion of additional individuals could also reduce the 95% confidence intervals for the relative risks

at other genotypes, potentially allowing genotype combinations with increased, but not high risk to be confirmed. Increasing the number of individuals genotyped may allow for more stringent age restrictions to be used in determining unaffected individuals which would increase the confidence in any associations made. In the current sample the 90% age restriction would be between 8 and 8.5 years for males, and 9 years for females. The use of these age restrictions in the current sample would reduce the number of individuals eligible to be included in the group to 13 which would reduce the power of the genetic association tests still further.

The physiological functions of the DCM/AF associated SNPs has not been confirmed, although some lie within or close to genes (Philipp et al., 2012). Of the three SNPs associated with DCM/AF in this current study only one, the SNP on chromosome 21 lies within a gene, *PDE3B* (Philipp et al., 2012). *PDE3B* has been shown to have a role in cardiac function so this intronic SNP has the potential to be a functional variant, or closely linked to a functional variant within the gene, although additional work is required to confirm this (Chung et al., 2015, Palmer and Maurice, 2000). Of particular interest is that there has been reported to be a difference in *PDE3B* mRNA expression between males and females (Wang et al., 2010). If *PDE3B* participates in the development of DCM/AF in IWHs, the differential expression between males and females could explain the difference in the age of onset between the sexes. There were not enough unaffected individuals in the current study to test the SNP for DCM/AF-association in each sex separately. With the inclusion of additional affected and unaffected individuals *PDE3B* could be a promising candidate gene for identifying the function of one aspect of the genetic basis of DCM in IWHs. The two other DCM/AF associated SNPs are not within known genes (Philipp et al., 2012). These SNPs could lie within regulatory regions or be in linkage disequilibrium with regulatory regions or functional genetic variants (Guo et al., 2014, Schaub et al., 2012). Further work is needed to establish the mode of action of these SNPs.

There are likely to be additional unidentified genetic susceptibility loci in IWH DCM/AF as there were affected individuals with genotypes which appeared to

confer a lower risk of disease (Table 4.12). In addition, there may be unidentified protective loci because there were unaffected individuals with genotypes conferring a higher risk of disease, although these individuals could develop disease later in life as the age restrictions for inclusion in the unaffected group were not very stringent. Further work with a larger number of individuals is required to verify the current genetic associations and identify additional loci associated with IWH DCM.

4.6. Conclusions

In conclusion, here we provide evidence that AF is related to DCM in the animals within this study and this novel information enabled individuals with both DCM and AF to be included in the affected group for genetic association testing. In addition male and female IWHs were confirmed to have different ages of disease onset which allowed more accurate allocation of individuals into the unaffected group. This study also confirms our previous work indicating that multiple loci analysis better predicts the incidence of DCM than single loci alone, however, a larger number of affected and unaffected individuals are required to confirm the associations.

Chapter 5 Differential gene expression in canine osteosarcoma

5.1. Abstract

Morbidity and mortality as a result of cancer is a concern globally contributing towards the social and financial costs of healthcare. Treatments and thus disease free intervals and cure rates for most cancers have been steadily improving. Osteosarcoma is one cancer where improvements in disease free intervals and cure rates have not improved in recent years. Improved understanding of the disease is required to develop novel treatments. Dogs also develop osteosarcoma and the disease process closely resembles that of human osteosarcoma which allows canine osteosarcoma to be used as a model of human disease. Osteosarcoma in dogs has incidence rates over 20 times higher than those in people and has a poor prognosis. In addition to canine osteosarcoma acting as a model for human disease due to the high incidence rates and poor prognosis it is also a veterinary clinical concern. Identifying genes and pathways that are differentially expressed in tumour tissue compared to non-tumour tissue could lead to targeted treatments. In this study the expression of genes in canine osteosarcoma tumour tissue was compared to the expression in non-tumour tissue utilising RNA sequencing with validation by qRT-PCR. There were 583 genes identified as differentially expressed between tumour (n = 4) and non-tumour tissue (n = 5). There was evidence from enrichment of transcription factor ontologies and validated gene expression that there was aberrant activation of the Wnt signalling pathway in canine osteosarcoma. Further work is warranted to validate these findings in a larger cohort and establish modes of action for the development of novel therapies.

5.2. Introduction

Cancer is a leading cause of mortality and morbidity, contributing towards the financial and social costs of healthcare, and associated loss of productivity within the workforce (Pearce et al., 2015, Siegel et al., 2016, WHO, 2014). Cancer is second only to cardiovascular disease in the number of adult deaths

between the ages of 30 and 70 years old (Siegel et al., 2016, WHO, 2014). Cancer typically affects adults, in particular older adults, whereas cancer in children and young adults is comparatively rare (Forman et al., 2014, Parkin et al., 2010). Despite the rarity of cancers in children and young adults some cancers are more prevalent in these age groups. Osteosarcoma is an example of such cancers.

Human osteosarcoma is a rare cancer that typically affects adolescents and the elderly (Mirabello et al., 2009a). Osteosarcoma accounted for 3,482 cancer cases in the USA SEER database between 1973 and 2004, compared to an estimated 217,440 breast cancer cases in 2004 alone (Jemal et al., 2004, Mirabello et al., 2009b). For most human cancers, 5 year survival rates have been steadily improving (Siegel et al., 2013, Siegel et al., 2015). In contrast, survival rates for osteosarcoma have seen little improvement in 20 years (Mirabello et al., 2009b, Siegel et al., 2013).

Improving osteosarcoma survival rates will only be possible with additional understanding of the disease and the development of novel treatments. There are some rare heritable syndromic conditions which increase the risk of developing osteosarcoma for which genetic bases have been established (Ognjanovic et al., 2012, Stinco et al., 2008, Thomas and Ballinger, 2015). There are risk factors for developing osteosarcoma including sex, race, puberty, and growth (Mirabello et al., 2009b, Mirabello et al., 2011, Savage et al., 2013). Although osteosarcoma itself does not generally appear to be heritable, the risk factors themselves have heritable components (Yang et al., 2010). To date there have been no external modifiable risk factors identified in the development of osteosarcoma outside of individuals with inherited cancer syndromes, where radiation has been identified as a factor in tumour development (Limacher et al., 2001, Villani et al., 2011). The lack of identified modifiable risk factors limits the development of effective osteosarcoma prevention strategies, thus to date the emphasis has been placed on the development of early diagnostic and improved treatment approaches to improve osteosarcoma outcomes in people.

One of the most effective strategies to improve understanding of disease processes and to develop new treatments is to compare affected and unaffected individuals (Betz et al., 2015, Eckstein et al., 2013, Mariani et al., 2013). In addition, animal models of disease, cell lines, and computer simulations can advance the understanding of disease processes (Al-Mamun et al., 2016, Brown et al., 2015, Lee et al., 2015, Simoni et al., 2002). In this chapter data will be presented from a natural animal model of osteosarcoma, that of canine osteosarcoma.

Canine osteosarcoma has several features that can be used to accelerate the understanding of the molecular basis of osteosarcoma, potentially facilitating more rapid development of novel diagnostic and therapeutic targets relevant to both people and dogs. These advantages include natural disease progression, a shared environment with people, and a higher incidence rate, along with a shorter lifespan resulting in a quicker clinical course. Canine osteosarcoma occurs naturally, reflecting the development of human osteosarcoma (Fenger et al., 2014). Other animal models of osteosarcoma rely on chemical induction, xeno/allografts, and genetically engineered animals which are unlikely to reflect many aspects of naturally occurring disease (Berlin et al., 1993, Fenger et al., 2014, Jacks et al., 1994, Tinkey et al., 1998). Most dogs are treated as family members within a household so inhabit the same environment as their owners and as such are exposed to many of the same environmental effects that could be implicated in osteosarcoma development, for example pollution (Calderón-Garcidueñas et al., 2008).

The incidence of canine osteosarcoma is higher than human osteosarcoma which provides additional cases to examine for genetic and environmental associations with disease. Between 1995 and 2002, 764 out of 394,061 insured Swedish dogs developed a bone tumour (Egenvall et al., 2007). This equates to an incidence rate of 27.2 dogs per 100,000 included in the dataset each year. In the comparable Swedish human population 234 men and 164 women had bone cancer between 1998 & 2002 (Parkin et al., 2010). This is roughly 0.89 cases of bone cancer per 100,000 people each year. Combined with exposure to similar environmental effects and natural disease progression the higher

incidence of osteosarcoma in the canine population compared to the human population makes the canine population an ideal model of human disease. In addition to being a good model for human disease, canine osteosarcoma is a clinical challenge in its own right. One-year survival rates following diagnosis and treatment are typically lower than 45% (Frimberger et al., 2016, Moore et al., 2007, Straw et al., 1991). Canine osteosarcoma typically affects large breeds, with Irish Wolfhounds, Rottweilers, St Bernards and Great Danes particularly affected (Egenvall et al., 2007).

Cancer results in aberrant cellular growth due to the disruption of normal cellular processes (Hanahan and Weinberg, 2000). While external factors have a role in cellular processes, it is the convergence of all environmental, endocrine, paracrine, autocrine, and receptor mediated signaling factors on genome function determines cellular identity, function and behaviour (Elrick and Docherty, 2001, Lin et al., 2016, Rajasekhar et al., 2003). Many cancers exhibit genome instability and have a high number of genetic mutations, including in tumour suppressor genes (Hubbard et al., 2016, Kantidakis et al., 2016, Li et al., 1997a, Ling et al., 2013, Popova et al., 2016, Srivastava et al., 1990). These mutations can lead to differential expression of genes in tumour compared to the equivalent normal tissue (Calon et al., 2015, Fehrmann et al., 2015, Gillet et al., 2016, van 't Veer et al., 2002). If these changes in gene expression can be identified and assessed to be critical for tumour growth, drugs targeting particular pathways could be utilised to treat cancers (Calon et al., 2015, Pfefferle et al., 2016, The Cancer Genome Atlas Research Network, 2014). Microarray expression profiling has been used to identify genes that are differentially expressed in osteosarcoma (Yang et al., 2014b). RNA sequencing is an alternative technology that utilises the whole mRNA transcript to identify genes that are differentially expressed between samples (Mortazavi et al., 2008). RNA sequencing has fewer limitations than expression microarrays with the advantage of being able to identify previously unidentified exons (Mortazavi et al., 2008).

It was hypothesised that RNA sequencing would identify novel differentially expressed genes in osteosarcoma tumour tissue as compared to matched non-

tumour tissue. This was carried out utilising canine osteosarcoma and associated non-tumour tissue.

5.3. Methods

5.3.1. Samples

This study was approved by the University of Nottingham ethics committee in compliance with Home Office regulations and the Veterinary Surgeons Act. Informed consent was obtained from all dog owners involved in this research. Osteosarcoma tumour and non-tumour tissue was obtained from a total of 7 individuals, four Rottweilers and three Deerhounds. Osteosarcoma tumour samples were collected by vets at the practices where patients were treated. These were either collected as part of treatment during amputation or immediately following euthanasia. A ~1cm^3 piece of tumour was extracted along with a ~1cm^3 piece of adjacent non-tumour affected tissue. These samples were placed directly into RNA*later*® (Sigma-Aldrich, USA) and stored at room temperature during shipping to the lab, upon arrival the samples were stored at -20°C.

5.3.2. RNA extraction

RNA was extracted from osteosarcoma tumour and associated non-tumour tissue samples using Qiagen RNeasy Mini Kit (Qiagen, Germany). For each RNA extraction approximately 350mg of tissue was cut into small pieces using a sterile scalpel before placing in a GentleMACS tube (Miltenyi Biotec, Germany) with 1mL of TRIzol® (Thermo Fisher Scientific, USA). The tissue was homogenised using a GentleMACS dissociator (Miltenyi Biotec, Germany) at 1,000rpm for 1 minute and the homogenate was then transferred to a 1.5mL microcentrifuge tube (Eppendorf, Germany). The homogenate was incubated at room temperature (15-25°C) for 5 minutes before adding 200µL chloroform (Sigma-Aldrich, USA). The homogenate/chloroform mixture was shaken vigorously for 15 seconds to fully mix, before incubating at room temperature for 2-3 minutes and then centrifuging at 12,000x g for 15 minutes at 4°C. The upper aqueous phase was transferred to a new 1.5mL microcentrifuge tube and 1 volume of 70% ethanol was added followed by thorough mixing by vortexing.

The RNA sample (600µL) was then transferred to the RNeasy Mini spin column placed in a 2mL collection tube which was centrifuged at 8,000x g for 15 seconds and the flow through was discarded. The remaining sample was transferred to the RNeasy Mini spin column and centrifuged at 8,000x g for 15 seconds and the flow through discarded. On column DNase digestion was performed. Firstly 350µL of Buffer RW1 was added to the RNeasy Mini spin column and centrifuged at 8,000x g for 15 seconds in order to wash the membrane. 10µL of DNase I was added to 70µl of Buffer RDD in a clean 1.5mL mircocentrifuge tube, this was added directly to the RNeasy Mini spin column membrane and incubated at room temperature for 15 minutes. 350µL of Buffer RW1 was then added to the RNeasy Mini spin column and then centrifuged at 8,000x g for 15 seconds. Buffer RPE (500µL) was added to the RNeasy Mini spin column and centrifuged at 8,000x g for 15 seconds to wash the membrane, the flow through was discarded. This 500µL Buffer RPE step was repeated and the flow through and collection tube were discarded. The RNeasy Mini spin column was the placed in a new collection tube and centrifuged at 17,000x g for 1 minute, the flow through and collection tube were discarded. The RNeasy Mini spin column was then placed in a clean 1.5mL microcentrifuge tube and 33µL of RNase free H_2O was added directly to the membrane. To elute the RNA from the membrane this was centrifuged at 8,000x g for 1 minute. To maximise yield the eluate was then added to the membrane and centrifuged at 8,000x g again. The extracted RNA was stored at -80°C.

5.3.3. RNA quantification

RNA was quantified using both a NanoDrop 8000 (Thermo Fisher Scientific, USA), and an Agilent 2100 Bioanalyzer (Agilent, USA). The Agilent 2100 Bioanalyzer is only accurate for RNA concentrations between 25 and 500ng/µL so based on the NanoDrop 8000 results the RNA was diluted appropriately prior to Agilent 2100 Bioanalyzer analysis.

5.3.4. RNAseq

RNAseq was completed at Edinburgh Genomics using an Illumina HiSeq platform. In all cases >20 million reads were obtained from all samples analysed. The resultant raw fastq reads were processed for quality (phred score

>30) and adapter sequences removed using Trimgalore. The processed raw reads were aligned to the canine genome (CanFam3.1) using the Ensembl annotated iGenomes build using Tophat2 (Kim et al., 2013a). Differential gene expression analysis was completed using Cufflinks/Cuffdiff (Trapnell et al., 2013). The RNAseq differential gene expression analysis was conducted by Dr. N. Mongan. The output of this differential gene expression analysis is available for examiners, but is not included in this thesis because of a confidentiality agreement between the University of Nottingham and a commercial entity. A subset of genes were selected for qPCR validation based on implication in cancer, biological function, statistical significance ($p<0.05$), and a fold change >2.

5.3.5. Quantitative Reverse Transcriptase PCR

cDNA was generated from the RNA extracted from osteosarcoma tumours and associated non-tumour tissue by reverse transcriptase PCR. The reverse transcriptase PCR reaction mixture consisted of 2µL of 5x qScript™ cDNA SuperMix (Quanta Biosciences, USA), 0.5-1µg RNA, adjusted to 10µL using PCR grade H_2O. Reverse transcriptase PCR was carried out in a water bath (Sub Aqua Pro, Grant Instruments, UK) at 25°C for 5 minutes followed by 42°C for 1 hour.

Gene expression of *MMP3*, *DKK3*, and *SLC2A1* was quantified using qRT-PCR. An additional 6 genes identified by RNAseq have been validated (N. Mongan personal communication). The reaction mixture consisted of 0.65µL TaqMan hydrolysis probe and primers, 7.5µL 2x Roche Probe Master Mix (Roche, Switzerland), 4.85µl H_2O, 2µl cDNA. Each sample was run in triplicate on a LightCycler 480 (Roche, Switzerland) PCR machine. Quantification of expression was performed using LightCycler 480 software (Roche, Switzerland). Expression for all samples were normalised to the internal control Actin (Actin qRT-PCR performed by Dr. N. Mongan) and relative expression of tumour compared to non-tumour tissue was calculated utilising the methods described by Pfaffl (2001). *t*-tests were performed using GraphPad Prism 7.01 for Windows (GraphPad Software, La Jolla California, USA) to establish if there

were significant differences in gene expression between the tumour and non-tumour tissue.

Gene ontology, KEGG pathway, Wiki Pathway, and transcription factor target analyses were performed by WebGestalt software to establish whether there were differentially expressed genes with common functions (Wang et al., 2013a, Zhang et al., 2005). These analyses were carried out utilising all genes identified as statistically significantly different ($p<0.05$) between tumour and non-tumour tissue, and with a fold change >2.

5.4. Results

5.4.1. Osteosarcoma RNAseq analysis

The Illumina iGenomes CanFam3.1 Ensembl annotated genome composed of 24,580 genes were included in the RNAseq analysis. Of these genes, 583 were identified with a fold change of greater than 2 between the tumour and non-tumour tissue and a p value of less than 0.05 in a comparison of five non-tumour samples as compared to four tumour samples. A total of 190 genes were more highly expressed in the tumour tissue compared to the non-tumour tissue, and expression of 393 genes was lower in the tumour tissue compared to the non-tumour tissue.

Gene ontology analysis identified five ontologies all from the biological processes category (q value <0.01): tetrapyrrole metabolic process, porphyrin-containing compound metabolic process, tetrapyrrole biosynthetic process, heme metabolic process, and pigment metabolic process (Table 5.1, Figure 5.1). KEGG pathway analysis identified two ontologies, porphyrin and chlorophyll metabolism, and metabolic pathways (Table 5.2). Wiki pathway analysis also identified two ontologies, heme biosynthesis, and calcium regulation in the cardiac cell (Table 5.3). All the genes in the gene ontology analysis, KEGG pathway analysis, and wiki pathway analysis ontologies were expressed less in the tumour than non-tumour tissue. There were 93 transcription factor targets with enriched ontologies in the dataset (Table 5.4). Of interest due to their association with cancer progression and metastasis, and association with genes chosen for validation by qRT-PCR were the following

transcription factors: *IRF8* (p = 0.0041), *LEF1* (p = 0.0000011), and *FOXO4* (p = 0.0002). *IRF8* (AKA *ICSBP*) had a total of 13 genes identified as differentially expressed associated with it (Table 5.4). Of these *IRF8* regulated genes 6 were more highly expressed in the tumour compared to non-tumour tissue, and 7 were more highly expressed in the non-tumour than the tumour tissue. *IRF8* expression was not significantly different between the tumour and non-tumour tissue in the RNAseq data (p = 0.16). *LEF1* expression was significantly different between tumour and non-tumour tissue (p = 0.034) with higher expression in tumour than non-tumour tissue. There were total of 70 genes associated with *LEF1* that were differentially expressed (Table 5.4), 30 of these were more highly expressed in the tumour tissue than the non-tumour tissue, while 40 were more highly expressed in the non-tumour tissue than the tumour tissue. *FOXO4* expression was significantly less in tumour compared to non-tumour tissue in the RNAseq data (p = 0.031). There were 64 genes differentially expressed that were associated with *FOXO4* (Table 5.4), of these 27 were more highly expressed in the tumour tissue than the non-tumour tissue and the other 37 were more highly expressed in the non-tumour tissue than the tumour tissue.

5.4.2. Osteosarcoma qRT-PCR validation

Three genes that were identified as differentially expressed in the RNAseq analysis were taken forward for validation by qRT-PCR. This confirmed the results of the RNAseq analysis with expression in the tumour tissue higher than the non-tumour tissue for *MMP3* (t = 2.48, p = 0.011), *DKK3* (t = 3.26, p = 0.0020), and *SLC2A1* (t = 4.47, p = 0.0001) (Figure 5.2).

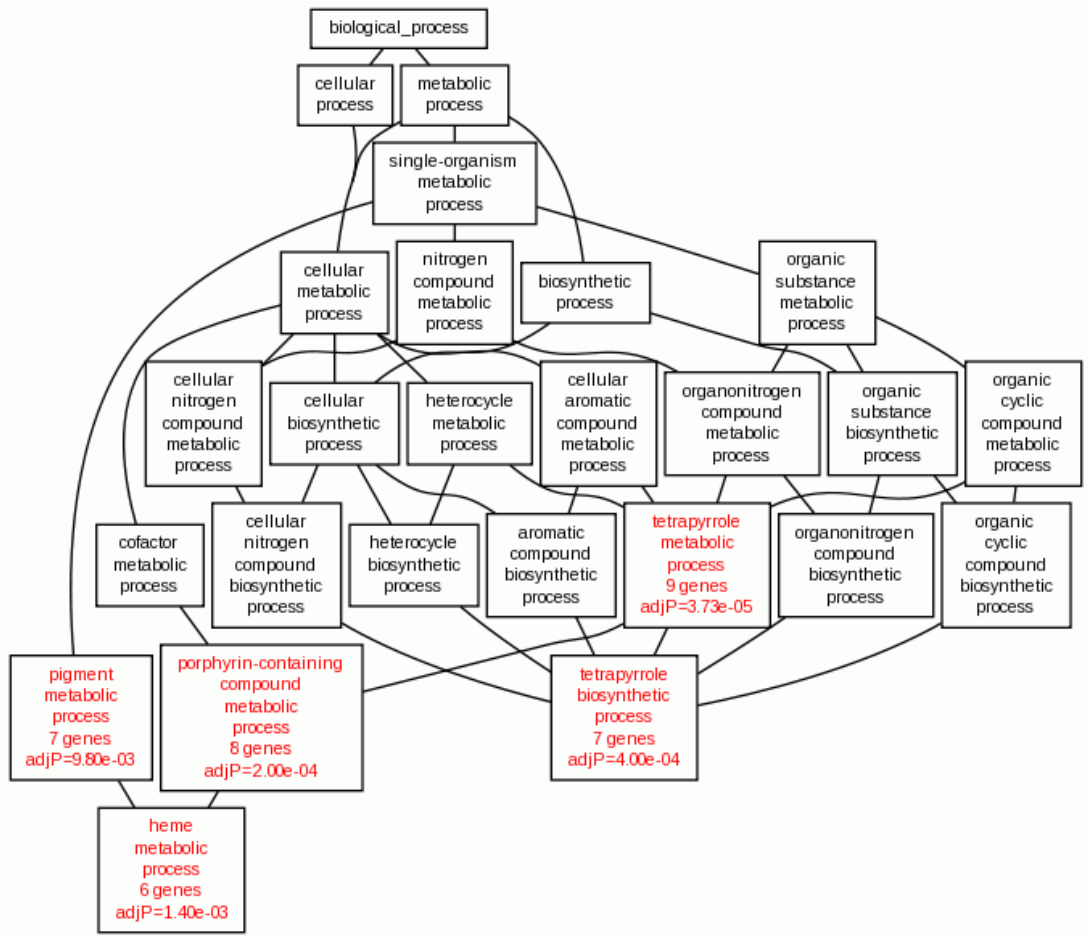


Figure 5.1. WebGestalt Directed Acyclic Graph showing gene ontology (GO) terms that are enriched in the current dataset. GO terms that are significantly enriched are shown in red. Enriched GO terms are involved in tetrapyrrole/heme/pigment metabolic and biosynthetic processes.

Table 5.1. Gene ontology (GO) terms identified as significantly (q<0.01) enriched in the dataset. Analysis was performed using WebGestalt. Enriched GO terms are involved in tetrapyrrole/heme/pigment metabolic and biosynthetic processes

Ontology	Name	Category	Genes in Ontology	Observed	Expected	Enrichment	q value
GO:0033013	tetrapyrrole metabolic process	BP	23	9	0.76	11.81	3.73E-05
GO:0006778	porphyrin-containing compound metabolic process	BP	22	8	0.7	11.45	0.0002
GO:0033014	tetrapyrrole biosynthetic process	BP	18	7	0.57	12.25	0.0004
GO:0042168	heme metabolic process	BP	15	6	0.48	12.6	0.0014
GO:0042440	pigment metabolic process	BP	30	7	0.95	7.35	0.0098

Table 5.2. KEGG Pathways identified as significantly (q<0.01) enriched in the dataset. Analysis was performed using WebGestalt. Enriched pathways include porphyrin metabolism and metabolic pathways.

KEGG Pathway Name	Genes in Ontology	Observed	Expected	Enrichment	q value
Porphyrin and chlorophyll metabolism	29	9	0.55	16.45	2.01E-07
Metabolic pathways	1103	42	20.81	2.02	0.0007

Table 5.3. Wiki Pathways identified as significantly (q<0.01) enriched in the dataset. Analysis was performed using WebGestalt. Enriched pathways include heme biosynthesis and calcium regulation.

Wiki Pathway Name	Genes in Ontology	Observed	Expected	Enrichment	q value
Heme Biosynthesis	8	6	0.15	39.76	5.71E-08
Calcium Regulation in the Cardiac Cell	143	11	2.7	4.08	0.0021

Table 5.4. Known transcription factors predicted to regulate differentially expressed genes identified in osteosarcoma identified as significantly (q<0.01) enriched in the dataset. Analysis was performed using WebGestalt. A range of transcription factors are enriched. The table is ordered by increasing q value.

Transcription Factor	Genes in Ontology	Observed	Expected	Enrichment	q value
E12	2308	91	43.54	2.09	5.60E-09
AP1	1036	50	19.54	2.56	2.63E-07
AP4	1395	59	26.32	2.24	8.46E-07
LEF1	1820	70	34.33	2.04	1.13E-06
NFAT	1727	64	32.58	1.96	1.30E-05
MEIS1	762	37	14.37	2.57	1.30E-05
TATA	1143	48	21.56	2.23	1.36E-05
CHX10	739	36	13.94	2.58	1.41E-05
ETS2	1012	44	19.09	2.3	1.43E-05
MMEF2	250	19	4.72	4.03	1.53E-05
CEBPB	243	18	4.58	3.93	3.88E-05
STAT6	243	18	4.58	3.93	3.88E-05
TATA	222	17	4.19	4.06	4.59E-05
SP1	2740	86	51.69	1.66	5.43E-05
PITX2	235	17	4.43	3.83	8.80E-05
MEF2	214	16	4.04	3.96	0.0001
MAZ	2118	69	39.95	1.73	0.0002
CEBPA	232	16	4.38	3.66	0.0002
FOXO4	1909	64	36.01	1.78	0.0002
RSRFC4	336	20	6.34	3.16	0.0002
TCF11MAFG	193	14	3.64	3.85	0.0004
MYC	964	38	18.19	2.09	0.0004
MEF2	223	15	4.21	3.57	0.0005
BACH2	251	16	4.73	3.38	0.0005
GATA	280	17	5.28	3.22	0.0005
AP1	256	16	4.83	3.31	0.0006
P53	232	15	4.38	3.43	0.0007
MYOD	861	34	16.24	2.09	0.0008
MYCMAX	236	15	4.45	3.37	0.0008
BACH1	240	15	4.53	3.31	0.0009
AML	240	15	4.53	3.31	0.0009
NFE2	246	15	4.64	3.23	0.0011
TCF11MAFG	276	16	5.21	3.07	0.0012
FOX	196	13	3.7	3.52	0.0013
TBP	223	14	4.21	3.33	0.0013
SMAD3	226	14	4.26	3.28	0.0013
IRF	227	14	4.28	3.27	0.0013
FREAC2	856	33	16.15	2.04	0.0013
LEF1	1109	39	20.92	1.86	0.0019
AP4	238	14	4.49	3.12	0.0019
AP1	241	14	4.55	3.08	0.0019
E47	239	14	4.51	3.11	0.0019
SRF	211	13	3.98	3.27	0.0019
LXR	86	8	1.62	4.93	0.0019

ERR1	959	35	18.09	1.93	0.0019
MYOD	240	14	4.53	3.09	0.0019
HNF3	675	27	12.73	2.12	0.0019
MAX	241	14	4.55	3.08	0.0019
SRF	207	13	3.9	3.33	0.0019
LBP1	208	13	3.92	3.31	0.0019
AMEF2	237	14	4.47	3.13	0.0019
HLF	240	14	4.53	3.09	0.0019
ZID	241	14	4.55	3.08	0.0019
TEF1	342	17	6.45	2.64	0.0025
PAX4	221	13	4.17	3.12	0.0025
FREAC4	139	10	2.62	3.81	0.0025
NKX25	246	14	4.64	3.02	0.0025
PU1	475	21	8.96	2.34	0.0025
CHOP	218	13	4.11	3.16	0.0025
ETS2	256	14	4.83	2.9	0.0032
E2A	227	13	4.28	3.04	0.0032
NFAT	227	13	4.28	3.04	0.0032
NKX22	173	11	3.26	3.37	0.0037
NRF2	231	13	4.36	2.98	0.0037
MYCMAX	233	13	4.4	2.96	0.0037
TGIF	230	13	4.34	3	0.0037
AP3	230	13	4.34	3	0.0037
IRF8	235	13	4.43	2.93	0.0041
FREAC2	238	13	4.49	2.9	0.0041
IRF	177	11	3.34	3.29	0.0041
GTF2A1	237	13	4.47	2.91	0.0041
PAX	236	13	4.45	2.92	0.0041
SRY	237	13	4.47	2.91	0.0041
TCF4	214	12	4.04	2.97	0.0055
LMO2COM	246	13	4.64	2.8	0.0059
AP1	246	13	4.64	2.8	0.0059
FOXJ2	162	10	3.06	3.27	0.0072
HNF3ALPHA	193	11	3.64	3.02	0.0076
AREB6	715	26	13.49	1.93	0.0076
CEBP	255	13	4.81	2.7	0.0076
NMYC	259	13	4.89	2.66	0.0086
ELF1	229	12	4.32	2.78	0.0086
TATA	262	13	4.94	2.63	0.0091

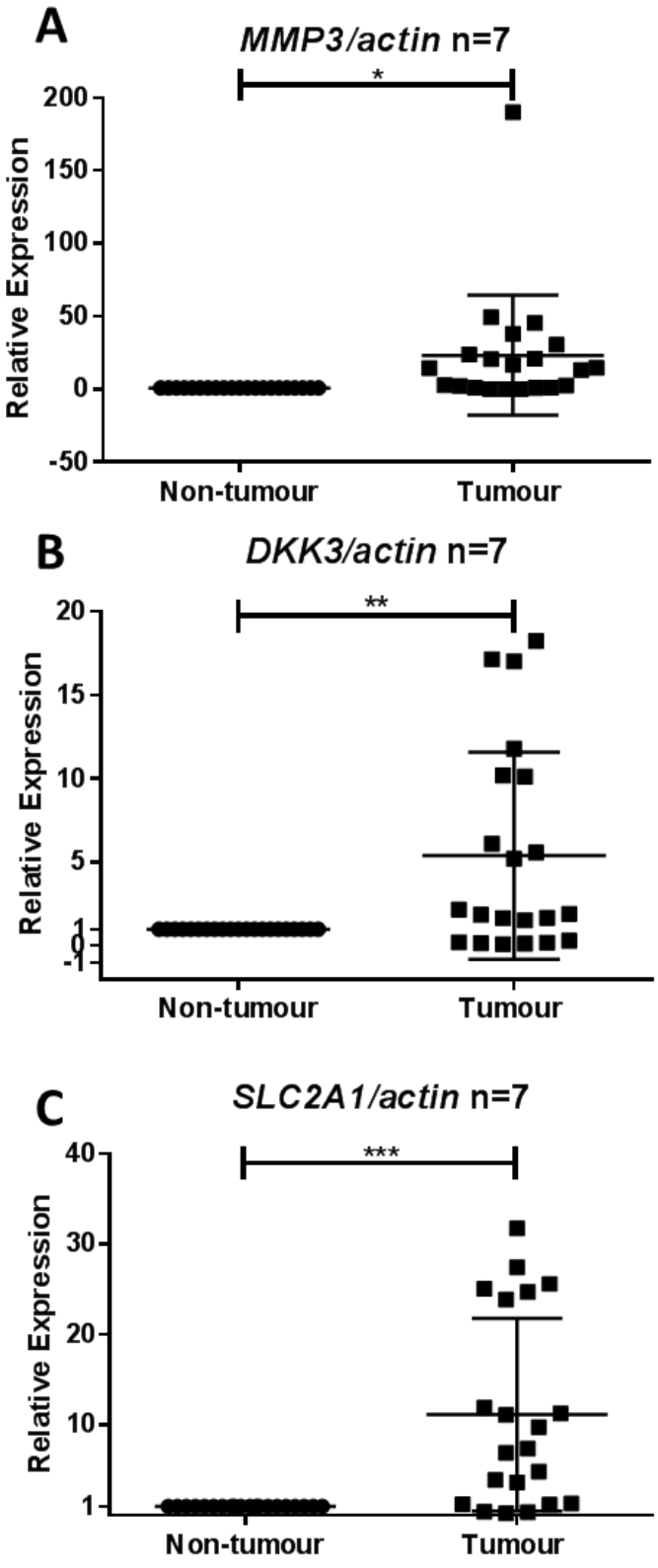


Figure 5.2. qRT-PCR validation of RNA expression comparing relative osteosarcoma tumour (Tumour) tissue expression with non-tumour (Non-tumour) bone tissue expression. Mean and standard deviation are shown. Results from *MMP3*, *DKK3*, and *SLC2A1* were consistent with the RNAseq results. t-test result significance levels * = p <0.05, ** = p <0.01, *** = p <0.001

5.5. Discussion

In cancers, differential gene expression has been associated with disease progression and prognosis (Calon et al., 2015, Fehrmann et al., 2015, Gillet et al., 2016, van 't Veer et al., 2002). This study examined differential gene expression in canine osteosarcoma and identified 583 genes as differentially expressed between tumour (n=4) and non-tumour (n=5) tissue by RNAseq analysis. Many of these genes had previously been identified as differentially expressed in other cancers and could be potential drug targets in the treatment of osteosarcoma. Three genes were chosen for qRT-PCR validation of the RNAseq results here, *MMP3*, *DKK3*, and *SLC2A1*. As noted earlier a further 6 genes have been validated (N. Mongan personal communication).

The *MMP3* gene encodes a member of the matrix metalloproteinase proteins which degrades a range of extracellular matrix components and is therefore an important mediator of metastatic invasion (Kurahara et al., 1999, Okada et al., 1986). Increased MMP3 protein expression has been associated with poor prognosis, and has been shown to be highly expressed in breast, lung, and pancreatic cancers (Mehner et al., 2015). In addition *MMP3* expression in the murine model of mammary carcinoma was important for primary tumour and metastasis growth (Banik et al., 2015). *MMP3* expression could be important for osteosarcoma, as the formation of metastases is a critical stage in disease progression associated with poor prognosis (Bielack et al., 2002, Reddy et al., 2015). Inhibition of MMP3 in individuals diagnosed with osteosarcoma could improve prognosis by reducing the likelihood of developing metastases. A selective inhibitor of MMP3 is available (UK370106), but this has not yet been tested for any potential to inhibit primary tumour or metastatic tumour growth (Fray et al., 2003). In addition to the MMP3 specific inhibitor there is a generic MMP inhibitor available (marimastat) (Bramhall et al., 2002). Despite the potential for marimastat to restrict tumour growth it has not been shown to be effective in clinical trials (Bramhall et al., 2002, Davies et al., 1993, Shepherd et al., 2002, Watson et al., 1999). In clinical trials of marimastat in pancreatic cancer and small-cell lung cancer it was not established whether the individuals enrolled in the trials had tumours expressing high levels of *MMP*s, if they did not this could have had implications for establishing an effect of the drug

(Bramhall et al., 2002, Shepherd et al., 2002). Marimastat has not been assessed for its potential to restrict primary tumour or metastatic tumour growth in osteosarcoma. In the present study it was shown that canine osteosarcoma expresses more *MMP3* than normal bone tissue (RNAseq p = 0.00005, qRT-PCR validation p = 0.012, Figure 5.2), this could make UK370106 and/or Marimastat potential candidates to improve the prognosis of canine osteosarcoma, although individuals should be assessed for expression of MMPs prior to inclusion in any clinical trials.

The current study is not the first to associate *MMP3* expression with osteosarcoma. Tsai *et al.* (2014) and Huang *et al.* (2016) found that *MMP3* was more highly expressed in osteosarcoma tumours than normal bone. In the study by Tsai *et al.* (2014) it was found that expression of an additional gene, *CTGF*, was associated with *MMP3* expression. In the current study *CTGF* expression was not significantly different between the tumour and non-tumour tissue (RNAseq p = 0.74), although it was expressed more highly in the tumour tissue than the non-tumour tissue. In addition to association with *CTGF* it has been shown that expression of *MMP3* is inversely related to expression of *IRF8* (Banik et al., 2015). Due to this, IRF8 could be an alternative drug target. In the current study expression of *IRF8* was not significantly different between the tumour and non-tumour tissue (RNAseq p = 0.16), however it was more highly expressed in non-tumour tissue than tumour tissue indicating a possible inverse relationship with *MMP3*. Interestingly *IRF8* is a transcription factor identified as significantly enriched within the RNAseq differentially expressed gene set (Table 5.4, p = 0.0041). This could indicate a role for *IRF8* in the development and progression of osteosarcoma. Although the expression of two genes previously shown to be regulators of *MMP3* are not significantly differentially expressed in the current study the direction of the non-significant differences between tumour and non-tumour expression were consistent with those expected if they were regulating *MMP3* in the current study. It is possible that these genes are expressed at higher levels in both the non-malignant and malignant bone of dogs predisposed to osteosarcoma, than in dogs without this predisposition. Given that this analysis only included dogs affected by osteosarcoma, it would not be possible to detect elevated expression of genes

which are constitutively higher in such dogs, as compared to unaffected dogs. It was not possible to quantify expression of these genes in unaffected dogs as it is not possible to secure tissue specimens from healthy dogs for ethical reasons. If the expression of *MMP3* can be validated as important in osteosarcoma progression, drugs targeting MMP3 directly could be employed, or alternatively drugs targeting key upstream regulators of *MMP3* could be utilised. Additional evidence for the role of MMP3 in osteosarcoma development comes from Adiguzel *et al.* (2016) in which polymorphisms within the *MMP3* gene were associated with osteosarcoma. The *MMP3* gene has not been assessed for polymorphisms in this dataset, but assessment of this gene may yield functional variants that predispose individuals to developing osteosarcoma.

A range of cancers have been shown to have aberrant Wnt activity (Dellinger et al., 2012, Dey et al., 2013, Voloshanenko et al., 2013, Woo et al., 2001). Dickkopf proteins inhibit Wnt signalling, and have been shown to be differentially expressed in colon cancer, prostate cancer, and breast cancer (Baehs et al., 2009, Hall et al., 2010, Zhou et al., 2010). Reduced *DKK3* expression has been shown in endometrial cancer, cervical cancer, and breast cancer, and as such it has been implicated as a tumour suppressor (Dellinger et al., 2012, Hsieh et al., 2004, Lee et al., 2009, Xiang et al., 2013). Within osteosarcoma there are conflicting reports with respect to *DKK3* expression. In osteosarcoma cell lines, and xenograft mice, *DKK3* expression was shown to be reduced, and subsequent restoration of *DKK3* expression reduced tumour and metastatic growth (Lin et al., 2013). Contrary to this in osteosarcoma tumour cells that overexpress *NKD2*, *DKK3* has been shown to be more highly expressed (Zhao et al., 2015). The results from the current study show that *DKK3* is more highly expressed in osteosarcoma tumour tissue than non-tumour tissue (RNAseq $p = 0.0045$, qRT-PCR validation $p = 0.0023$, Figure 5.2). This is in contrast to expression in most other cancers, but in agreement with Zhao *et al.* (2015), who found that osteosarcoma tumour cells that overexpress NKD2, DKK3 was more highly expressed. Knockdown of *DKK3* in cells overexpressing *NKD2* increased their proliferative potential indicating that *DKK3* expression could be mediating *NKD2* induced metastasis (Zhao et al.,

2015). There are currently no drugs available that act on DKK3, if DKK3 is further validated as important in osteosarcoma drugs acting on DKK3 could be developed.

MMP3 and DKK3 have been shown to have opposite effects on Wnt activation. MMP3 secretion has been shown to lead to increased Wnt activation (Kessenbrock et al., 2013, Kim et al., 2016a) while increased *DKK3* expression results in reduced Wnt activation (Dellinger et al., 2012, Hsieh et al., 2004, Lee et al., 2009, Xiang et al., 2013). The results presented in this current study thus appear contradictory with both *MMP3* and *DKK3* expressed more highly in tumour compared to non-tumour tissue (Figure 5.2). It is possible that DKK3 is ameliorating the effect of MMP3 secretion in the tumours in this study in a similar way to the effect identified by Zhao *et al.* (2015). Further work is required to establish the relationship between *MMP3* and *DKK3* in osteosarcoma tumours.

Glucose is an essential component in cellular metabolism, with transport into cells reliant on membrane glycoproteins (Shows et al., 1987). *SLC2A1* is a gene encoding one such cell membrane glycoprotein which has been shown to be involved in glucose transport in a range of tumours including oral squamous cell carcinoma, non-small cell lung carcinoma, and non-gastrointestinal stromal tumour soft tissue sarcomas (Kunkel et al., 2003, Smeland et al., 2012, Younes et al., 1997). In patients with osteosarcoma increased expression of *SLC2A1* within tumours has been associated with a shorter disease free interval, and poorer prognosis than patients with little or no expression of *SLC2A1* (Kubo et al., 2015). In the current study expression of *SLC2A1* is significantly higher in osteosarcoma tumour tissue as compared to non-tumour tissue (RNAseq p = 0. 012, qRT-PCR validation p = 0.0002, Figure 5.2). Survival time is not available for the patients involved in the current study, but canine osteosarcoma survival time is typically short, based on the current results combined with those of Kubo *et al.* (2015) this could be in part due to the high expression of *SLC2A1* (Frimberger et al., 2016, Moore et al., 2007, Straw et al., 1991). There are several drugs available that have inhibitory effects on cellular glucose transport and SLC2A1, but few have been tested as a treatment for osteosarcoma

(Klepper et al., 2003, Louters et al., 2010, Rufino et al., 2013, Shuralyova et al., 2004). If these drugs that have not been tested with regards to osteosarcoma could be established to inhibit SLC2A1 in osteosarcoma they could be additional drugs to treat osteosarcoma.

Significantly enhanced gene ontologies were identified in the current study (Figure 5.1, Tables 5.1 - 5.3). These include ontologies involved in tetrapyrrole/heme/pigment metabolic and biosynthetic processes (Figure 5.1, Tables 5.1 - 5.3). Further investigation showed that the genes in the ontologies were all expressed less in the tumour compared to non-tumour tissue. This is likely to be attributable to the loss of normal bone function as bone marrow is a major source of heme synthesis and haematopoiesis (Morell et al., 1958). An additional possibility is that the loss of heme synthesis within the tumour contributes to its progression. There is some evidence that tetrapyrroles, of which heme is one, possess anti-cancer properties by inducing apoptosis (Mölzer et al., 2013). The products of the catalysis of heme by heme oxygenases, biliverdin and bilirubin, have been shown to have anti-oxidant properties (Jansen et al., 2010). Due to the reduced expression of genes involved in tetrapyrrole/heme/pigment metabolic and biosynthetic processes in tumour compared to non-tumour tissue this study it would seem likely that the apoptosis and anti-oxidant properties of tetrapyroles is correspondingly reduced. It is not possible in this study to establish if this is as a result of the tumour, or a cause of the cancer, but further investigation could establish additional potential drug targets acting on tetrapyroles.

There were a number of transcription factor targets which were significantly enriched in the RNAseq data (Table 5.4). Of interest were *IRF8*, *LEF1*, and *FOXO4*. *IRF1* is interesting due to its implicated role in the regulation of *MMP3*, as discussed above (Banik et al., 2015).

LEF1 was significantly differentially expressed in the current study with higher expression in tumour than non-tumour tissue in the RNAseq data ($p = 0.034$), in addition as a transcription factor it was significantly enriched (Table 5.4, $p = 0.0000011$). Within the genes that are regulated by *LEF1* some had expression

increased expression in the tumour compared to non-tumour tissue, but others had decreased expression. Genes do not act in isolation, therefore those genes which had a lower expression in this dataset may also have required the expression of additional transcription factors or coactivators for activation, although it was not possible to determine this in the current study (Shakya et al., 2015).

LEF1 is associated with increased Wnt signalling, however in this study it appears that there may be genes acting against each other, with genes that both increase and decrease Wnt signalling expressed more highly in the tumour compared to non-tumour tissue (Figure 5.2) (Kessenbrock et al., 2013, Kim et al., 2016a, Reya et al., 2000, Zhao et al., 2015). Wnt signalling has both canonical and non-canonical pathways, with non-canonical signalling split into planar cell polarity and Wnt/Ca^{2+} (Huelsken and Behrens, 2002). DKK3 and MMP3 act on the binding of Wnt activator proteins so alterations in the amount of these proteins will affect both canonical and non-canonical Wnt signalling (Huelsken and Behrens, 2002, Kessenbrock et al., 2013, Nakamura and Hackam, 2010). *LEF1* is a key protein in the Wnt signalling pathway that appears to have aberrant activation in osteosarcoma tumours compared to non-tumour bone tissue in this study. Combined with the evidence from *DKK3* and *MMP3* expression (Figure 5.2) the aberrant activation of *LEF1* adds osteosarcoma to the range of cancers that have been shown to have aberrant Wnt activity (Dellinger et al., 2012, Dey et al., 2013, Voloshanenko et al., 2013, Woo et al., 2001). LEF1 is part of the canonical Wnt signalling pathway, requiring β-catenin as a coactivator for downstream gene transcription (Behrens et al., 1996, Hsu et al., 1998). It may be that aberrant Wnt processes upstream of LEF1 such as those of DKK3 and MMP3 are affecting LEF1 activity which may explain the difference in transcription of genes regulated by LEF1, but not LEF1 itself in osteosarcoma tumour tissue compared to non-tumour tissue. LEF1 could be an additional drug target to influence Wnt signalling within osteosarcoma tumours. Prior to targeting *LEF1* further work is required to establish the effects that this would have on *LEF1* regulated genes identified as differentially expressed. Focussing on individual downstream genes may lead to more targeted treatment.

FOXO4 has been shown to be predictive of survival and disease free interval in gastric carcinoma, with higher expression associated with longer survival and disease free interval than lower expression (Li et al., 2016a). In addition up-regulation of *FOXO4* has been shown to inhibit the growth and progression to metastasis in gastric cancer (Su et al., 2014). In the current study *FOXO4* expression was significantly lower in tumour compared to non-tumour tissue in the RNAseq data ($p = 0.031$). This is consistent with the results of Li *et al.* (2016a) which indicate that low expression of *FOXO4* correlates with poor prognosis, and corresponds with the observations that osteosarcoma typically has poor prognosis (Frimberger et al., 2016, Mirabello et al., 2009b, Moore et al., 2007, Siegel et al., 2013, Straw et al., 1991). FOXO4 is a transcription factor, and it was identified as significantly enriched within the current study (Table 5.4, $p = 0.0002$). As with LEF1 not all FOXO4 enriched genes were expressed in the same direction with some more highly expressed in the tumour compared to the non-tumour tissue, and some more highly expressed in the non-tumour tissue than the tumour. It is not clear whether FOXO4 is the predominant transcription factor regulating these genes, but over half were expressed less in the tumour tissue compared to the non-tumour tissue indicating that for these genes with lower expression *FOXO4* could be the key regulator. As up-regulating *FOXO4* has been shown to inhibit the growth and progression to metastasis in gastric cancer cell lines *FOXO4* could be a potential drug target in the treatment of osteosarcoma (Su et al., 2014). NAD+ has been shown to upregulate *FOXO4* and has been associated with longevity in *C. elegans*, while additional work is required this could be a potential drug for the treatment of osteosarcoma in the future (Mouchiroud et al., 2013).

5.5.1. Conclusions

This study has identified several potential therapeutic targets for improving the outcomes of individuals with osteosarcoma. Additional work is required to validate these results in a larger study and to determine the functional/mechanistic importance of the difference in expression between tumour and non-tumour tissue. Drugs targeting some of the genes identified already exist thus these could be investigated for an effect in osteosarcoma easier than genes which do not have drugs available.

Chapter 6 Polyglutamine expansions in AR and NCOA3 genes are not associated with dilated cardiomyopathy, atrial fibrillation or osteosarcoma in Irish Wolfhounds and Rottweilers

6.1. Abstract

The androgen receptor is a ligand dependent transcription factor which contains a polymorphic polyglutamine tract. Individuals with shorter polyglutamine tracts are more sensitive to androgens than individuals with longer polyglutamine tracts. Variation in the length of this tract has been associated with a range of human diseases. The canine androgen receptor gene contains two polyglutamine tracts and variation within these tracts has been associated with aggression and prostate cancer in dogs. Binding of ligands to the androgen receptor is also dependent on coactivators such as NCOA3. NCOA3 also contains a polyglutamine tract with variation in length associated with disease in humans. The canine *NCOA3* gene also contains a polyglutamine tract, but variation in the length has not been examined for an association with disease. In this study the polyglutamine tracts in both the androgen receptor and *NCOA3* genes were examined for association with canine dilated cardiomyopathy and canine osteosarcoma in Irish Wolfhounds and Rottweilers. It was established that there is little variation in the canine *NCOA3* polyglutamine tract across both breeds, thus no associations were made between the length of the polyglutamine tract and either canine disease. Greater variation was present in both polyglutamine tracts in the canine androgen receptor, but no significant associations in the length of the polyglutamine tracts were made with either osteosarcoma or DCM/AF. This study does not exclude a role for the androgen receptor in the development of canine dilated cardiomyopathy or canine osteosarcoma, but it does exclude the role of the polyglutamine tracts in the androgen receptor and its coactivator *NCOA3*.

6.2. Introduction

Transcription factors are key regulators of gene expression (Odom et al., 2004, Tupler et al., 2001). The androgen receptor (AR) is a member of the ligand-

dependent superfamily of transcription factors (Fang et al., 2003, Lu et al., 2006, Mongan et al., 2015, Yang et al., 2016). In the presence of an agonist, the AR recruits many enzymatically distinct epigenetic coregulators, to mediate transcriptional regulation (McKenna and O'Malley, 2002). Notable examples of coregulators include the p160 coactivators, NCOA1, NCOA2 and NCOA3 (McKenna and O'Malley, 2002). The *AR* gene is X-linked, therefore both males and females both possess functional AR, but activation of the receptor differs between the sexes due to differing amounts of androgens (Dart et al., 2013). These differences between the sexes lead to phenotypic variation throughout life, including in *utero* (Evans et al., 2008, Hahn et al., 2016, Lamminmäki et al., 2012, Moore et al., 2001, Pangelinan et al., 2016). Androgens are the most abundant naturally occurring ligands of the AR, and although alternative and synthetic ligands exist they have limited clinical and research applications (Fang et al., 2003, Yang et al., 2016). Physiological androgens include testosterone, dihydrotestosterone, dehydroepiandrosterone, androstenedione, and androstenediol (Kushnir et al., 2010, Morales et al., 1998). Adult males have higher concentrations of testosterone, androstenediol, and dihydrotestosterone, and lower concentrations of androstenedione as compared to females (Dabbs, 1990, Kushnir et al., 2010, Morales et al., 1998, Rosenfield and Otto, 1972). However there are no differences between the sexes in the concentrations of dehydroepiandrosterone (Kushnir et al., 2010, Morales et al., 1998). Concentrations of testosterone in particular are between 5-13 fold higher in males than females (Dabbs, 1990, Kushnir et al., 2010, Morales et al., 1998).

Mutations in the *AR* gene can alter the sensitivity of the receptor to androgens (Gottlieb et al., 2012, Infante et al., 2016). Some *AR* mutations cause complete androgen insensitivity where genetically 46XY males appear to be phenotypically female (Batch et al., 1992, Brown et al., 1988, Infante et al., 2016, Mongan et al., 2015). Other *AR* mutations contribute to partial androgen insensitivity, with diverse clinical presentations including hypospadias, genital under-masculinization, and gynecomastia (Bevan et al., 1996, Bevan et al., 1997, Evans et al., 1997). In addition to loss of function *AR* mutations, there is a polymorphic polyglutamine tract within the *AR* gene which shows variation in

the number of polyglutamine repeats (Chamberlain et al., 1994, Kazemi-Esfarjani et al., 1995). Variation in the number of polyglutamine repeats in the *AR* gene has been associated with prostate cancer, breast cancer, ovarian hyperandrogenism, and Kennedy's disease (Giovannucci et al., 1997, Hsing et al., 2000, Ibanez et al., 2003, Rebbeck et al., 1999, Spada et al., 1991). Individuals with shorter polyglutamine tracts are more sensitive to androgens, while individuals with longer polyglutamine tracts are less sensitive to androgens (Chamberlain et al., 1994, Kazemi-Esfarjani et al., 1995).

Many cancers and heart diseases have a sex bias with males commonly affected more frequently, and/or earlier in life (Aguero et al., 2008, Egenvall et al., 2006, Egenvall et al., 2007, Mirabello et al., 2009a, Mozaffarian et al., 2016, Phillips et al., 2007, Wess et al., 2010). The differences in circulating androgens and the response to these hormones has been proposed as a contributor to this sex-disparity in disease incidence (Arimura et al., 2013, Dabbs, 1990, Dart et al., 2013, Kushnir et al., 2010, Morales et al., 1998, Rosenfield and Otto, 1972, Wagle et al., 2015). In the context of this thesis it is notable that both osteosarcoma and dilated cardiomyopathy have been shown to have a male bias in people and dogs (Bagger et al., 1984, Doesch et al., 2014, Egenvall et al., 2007, Mirabello et al., 2009a, Lin et al., 2015, Truszkowska et al., 2015). Indeed osteosarcoma is an example of a cancer which affects males more frequently than females (Egenvall et al., 2007, Mirabello et al., 2009a).

As outlined earlier osteosarcoma is a rare cancer that primarily affects adolescents and the elderly and notably outcomes have not improved in the past 20 years despite a general trend of improved prognosis following diagnoses of other cancer types (Mirabello et al., 2009a, Mirabello et al., 2009b, Siegel et al., 2013). In people most instances of osteosarcoma do not appear to be directly heritable, although there are some osteosarcoma cases associated with heritable cancer syndromes (Ognjanovic et al., 2012, Stinco et al., 2008, Thomas and Ballinger, 2015). Despite the lack of heritability there are some risk factors associated with the development of osteosarcoma, these include male sex, race, and growth (Mirabello et al., 2009b, Mirabello et al., 2011). Males are more frequently affected than females and there is some

evidence that there is variation in the incidence rate between different races in the USA with the highest incidence rate in African Americans (Mirabello et al., 2009b). Interestingly African Americans have been shown to have the highest frequency of short *AR* polyglutamine tracts, with short *AR* polyglutamine tracts associated with increased risk of prostate cancer (Giovannucci et al., 1997, Hsing et al., 2000, Nelson and Witte, 2002). It may be possible that these short *AR* polyglutamine tracts are also contributing to the higher incidence rate of osteosarcoma in African Americans (Mirabello et al., 2009b). In addition to sex and race, growth also appears to be involved in the development of osteosarcoma with affected individuals usually taller at diagnosis than unaffected individuals (Mirabello et al., 2011). Although osteosarcoma itself does not appear to be heritable, the risk factors themselves have heritable components (Yang et al., 2010). Canine osteosarcoma reflects the sex differences of human osteosarcoma with males affected more frequently than females (Egenvall et al., 2007). In addition there also appears to be a size difference with larger, taller breeds of dog affected more often than smaller breeds (Egenvall et al., 2007).

As reported earlier (Chapter 4) dilated cardiomyopathy (DCM) also has a similar male-sex bias and it is a disease with similar poor prognosis to osteosarcoma (Bagger et al., 1984, Doesch et al., 2014, Lin et al., 2015, Truszkowska et al., 2015). This is reflected in dogs with some breeds having a higher incidence of males developing DCM, and others having an age effect with females developing DCM as frequently, but later in life, than males (Martin et al., 2010, Wess et al., 2010) (Chapter 4).There is currently no cure for DCM and existing treatments seek to delay progression and minimize the effects of DCM on the patient's quality of life (Dukes-McEwan, 2000, Jefferies and Towbin, 2010). Ultimately DCM leads to heart failure and death (Egenvall et al., 2006, Lund et al., 2015).

While there are no known mechanistic or genetic links between osteosarcoma and DCM, it is possible there are common underlying molecular mechanisms underpinning the established sex disparity in incidence of osteosarcoma and DCM. In this chapter, the hypothesis that the AR-polyglutamine repeat length,

and by inference the responsiveness to androgens, is associated with incidence of osteosarcoma and DCM in dogs was tested. Variation in the length of the canine androgen receptor polyglutamine tract has previously been associated with aggression in Japanese Akita Inu and canine prostate cancer, but it has not been examined with respect to osteosarcoma or DCM (Konno et al., 2011, Lai et al., 2008). Thus the length of the canine polyglutamine tract in the *AR* gene was tested for an association with osteosarcoma and DCM in the Irish wolfhound (IWH) breed. IWHs are a giant breed of dog with a high incidence of both osteosarcoma and DCM (Egenvall et al., 2006, Egenvall et al., 2007). In addition to the development of DCM, previous work (Chapter 4) has shown that IWHs also develop atrial fibrillation (AF) and that the development of AF is frequently a precursor to DCM in the breed. To account for breed variation an additional dog breed, the Rottweiler, was assessed for an association with the length of the polyglutamine tract with OSA. The Rottweiler is a large breed of dog which has a high incidence of osteosarcoma (Egenvall et al., 2007).

Androgen activation of the AR requires the presence of coactivators (Boorjian et al., 2009, Gnanapragasam et al., 2001, Ngan et al., 2003, Yepuru et al., 2013). One such coactivator NCOA3 also harbours a polyglutamine repeat (Mongan et al., 2003, Tan et al., 2000). Expression of NCOA3 has been associated with increased AR activation in prostate cancer and urothelial carcinoma of the bladder (Boorjian et al., 2009, Gnanapragasam et al., 2001). In addition increased expression of NCOA3, with implications for androgen receptor activation, and variation in the length of the polyglutamine repeat in the *NCOA3* gene have been associated with bone cancer, epithelial ovarian cancer, colorectal cancer, and breast cancer (Burandt et al., 2013, Han et al., 2015, Li et al., 2012, Luo et al., 2013). For this reason in addition to assessing the length of the *AR* polyglutamine tract, the polyglutamine tract within the *NCOA3* gene was also examined for an association with osteosarcoma and DCM in Irish Wolfhounds and osteosarcoma in Rottweilers.

6.3. Methods

6.3.1. DNA samples

This study was approved by the University of Nottingham ethics committee in compliance with Home Office regulations and the Veterinary Surgeons Act. Informed consent was obtained from all dog owners involved in this research. Buccal swabs from 379 IWHs and 1019 Rottweilers were taken using Isohelix DNA Buccal Swabs (Cell Projects Ltd. UK). The swab was inserted into the dog's mouth by either the owner, vets, or trained members of the research group, and rubbed on the cheek for up to 2 minutes to enable optimum cell collection. Swabs were stored as directed by the manufacturers, at room temperature upon collection followed by 4°C. Any swabs stored for longer than a month prior to DNA extraction were stored at -20°C. Optimisation trials indicated that appropriate quality and quantity of DNA could be obtained following all storage methods. When the buccal swabs were taken owners completed a short form for each dog to obtain current and past health information, date of birth, pedigree information, sex, and neutering status, a copy of the form is shown in Appendix 3. All information was associated with a unique sample number and stored in a secure excel database.

Follow-up data on the health of sampled dogs was obtained at numerous time points. Owners were asked to fill in a 'health update' form annually or following any change in health/upon death. Forms could be completed either online or in paper format to ensure maximum owner participation, a copy of this form is in Appendix 3. In addition telephone surveys were carried out by undergraduate research project students who were supervised by the PIs on this canine health project. The surveys were utilised to obtain health updates and epidemiology data on all samples obtained. A copy of the questionnaire is shown in Appendix 4. Many IWHs are presented to specialist veterinary cardiologists on a regular basis to be screened for heart disease. These heart screenings consist of cardiac auscultation, a six lead electrocardiogram, and complete echocardiographic assessment. Data from these heart screenings were provided by veterinary by cardiology specialist Dr Serena Brownlee. Cardiac diagnoses were determined by Professor Malcolm Cobb based on the data from heart screenings. Information from owner health up-dates and heart

screenings was added to that stored in the excel database and analysed alongside genetic data.

All individuals with diagnoses of DCM/AF and/or osteosarcoma were identified in the database. The hypothesis that males are more frequently affected by osteosarcoma than females was tested by establishing the total number of affected and unaffected males and females in IWHs and Rottweilers separately. A χ^2 test was performed to establish whether there were significant differences in the number of males and females diagnosed with osteosarcoma. The age at diagnosis of osteosarcoma was established for all individuals. *t*-tests were performed using R statistical software (RCoreTeam, 2015) on the IWH and Rottweiler datasets separately to establish if there were statistical differences between males and females within the breeds in the age of onset. From this, appropriate age restrictions for each sex and breed were established for the inclusion of unaffected individuals in the unaffected control group, based on the age by which most affected individuals had been diagnosed. Individuals in the unaffected group were used in genetic association testing. Previous work has shown that there is a difference in age of onset of DCM/AF in IWHs and thus different age restrictions for inclusion of healthy individuals in the unaffected group were determined (Chapter 4). This study utilised the same age restrictions as previously to include IWHs in the unaffected by DCM/AF group - 6.5 years for males and 8.5 years for females.

6.3.2. DNA extraction

To extract DNA from buccal swabs, the end of the swab was first dissociated into small pieces using scissors which were sterilised between swabs using Anistel (Tristel, UK) and then wiped down with Industrial Methylated Spirits (IMS) (Thermo Fisher Scientific, USA). The small pieces of swab were put into a sterile 1.5mL microcentrifuge tube (Eppendorf, Germany). The DNA-containing swab fragments were re-suspended in 600µL of a solution composed of 5% sodium Chelex® 50-100 mesh (dry) (Sigma-Aldrich, USA) reconstituted in 1xTE (10mM Tris (Thermo Fisher Scientific, USA)), 1mM EDTA (Thermo Fisher Scientific, USA) in H_2O, pH 8.0) and 20µL of 10mg/mL

proteinase K (Promega, USA), 50mM Tris (Thermo Fisher Scientific, USA), 10mM $CaCl_2$ (Thermo Fisher Scientific, USA) pH 8.0. The sample-containing microcentrifuge tube was vortexed to mix the components. The microcentrifuge tube was then placed in a water bath at 45°C overnight to digest the cells. The sample was then vortexed once more before incubation in a heating block at 100°C for 8 minutes to inactivate the proteinase K. The sample was then centrifuged at 13,500xg for 3 minutes to separate the Chelex® and swab from the supernatant. The DNA-containing supernatant was then transferred into a sterile 1.5mL microcentrifuge tube and was used in subsequent PCRs. The extracted DNA was then stored at -20°C in the Nottingham Comparative Genomic Biobank.

6.3.3. Primers, PCR and fragment length analysis

Unlike in the human *AR* ortholog where the polyglutamine repeat is continuous, the canine *AR* harbours two polyglutamine repeat regions within the canine androgen receptor gene (Loci "*AR1*" and "*AR2*") (Maejima et al., 2005). *AR1* and *AR2* were PCR amplified using the primers published by Maejima *et al.* (2005). Primers were also designed (by Dr. N. Mongan) to flank the polyglutamine region of the canine *NCOA3* gene. One of each pair of primers was fluorescently labelled with HEX or 6-FAM dyes (Sigma-Aldrich, UK) to allow fragment analysis to be carried out. Primer sequence information is in Table 6.1.

Table 6.1. PCR primer sequences for androgen receptor loci *AR1* and *AR2*, and *NCOA3*.

Locus	Forward primer	Reverse primer
AR1	6-FAM-CCGTGAGCGCAGCACCTCCCGGTG	AGGCTGACCGCTGTTGGGAAGGCTGC
AR2	6-FAM-GCCAGCACCACCGGACGAGAATGA	TAACTGTCCTTGGAGGAGGTGGAAGCA
NCOA3	HEX-CCCAGCAGGGTTTTCTGAATGCCC	CACAGGCCCTGCCAAAACGCCATCC

Pre-PCR multiplexing whereby two PCR reactions are carried out on a sample in a single reaction was performed for *AR1* and *NCOA3* with no discernible effect on PCR efficiency. The reaction mixtures consisted of 1x LightCycler® 480 Probes Master Mix (Roche, Switzerland), primers at a concentration of 0.5µM, 1.5µL of template DNA per reaction, adjusted to 15µL

using PCR grade H_2O. The calculation for a 15µL reaction containing *AR1* and *NCOA3* primers combined was 7.5µL 2x Roche Probe Master Mix, 1.5µL of each primer from a stock with concentration of 5µM, and 1.5µL template DNA. *AR2* was added to the multiplex post PCR, the calculation for a 15µL reaction for the AR2 primer pair was 7.5µL 2x Roche Probe Master Mix, 1.5µL of each primer from a stock with concentration of 5µM, 3µL H_2O, and 1.5µL template DNA. PCR reactions were carried under the following conditions: Initial denaturation 94°C for 30 seconds, 40 cycles of: 94°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 30 seconds, final extension 72°C for 5 minutes. The annealing temperature optimisation was determined by performing a temperature gradient PCR between 52°C and 64°C. An annealing temperature of 61°C was used for *AR1* and *NCOA3*, and 57°C was used for *AR2*.

All three fluorescently labelled PCR fragments were detected using an Applied Biosystems 3730 DNA Analyzer with GeneScan ROX-500 size standard (DBS Genomics, Durham, U.K.) in a single multiplex. This was possible because the FAM labelled PCR products were approximately 100bp different from each other. The multiplex consisted of the fluorescently labelled PCR fragments at a 1 in 100 dilution. Genotypes were scored using Genemapper software v3.7 (Applied Biosystems, USA). A homozygote of each allele was Sanger sequenced by Source Bioscience (Nottingham, UK) to confirm the number of polyglutamine repeats.

6.3.4. *AR* and *NCOA3* associations with disease

The length of *AR1* and *AR2* were combined to give an overall *AR* polyglutamine repeat tract length for each individual. The *AR* gene is on the X chromosome, males only have one copy of the gene while females have two. For males the number of repeats in *AR1* and *AR2* were added together, while for females the mean of the two was established and then added together. The mean length of the *NCOA3* polyglutamine tract was established for all individuals. *t*-tests were performed using R statistical software (RCoreTeam, 2015) to establish whether there were differences between groups in the length of the polyglutamine repeat in *AR* (combined *AR1* and *AR2*) and *NCOA3*. *AR1* and *AR2* tested for

association with disease separately. The groups that were compared were IWH DCM/AF vs not DCM/AF, IWH osteosarcoma vs not osteosarcoma, and Rottweiler osteosarcoma vs not osteosarcoma. The individuals included in the unaffected groups were established by the age restrictions as determined above. The groups were split further by sex for each breed and diagnosis and were then analysed by ANOVA using R statistical software (RCoreTeam, 2015).

The frequency of the *AR1*, *AR2*, and *NCOA3* alleles was established utilising all genotyped individuals irrespective of disease status and χ^2 tests for each locus were performed to establish if there were significant differences between the breeds. The allele frequencies for each locus were visualised on bar charts by breed.

6.4. Results

There were 12 IWHs (8 females and 4 males) diagnosed with osteosarcoma, representing 3.2% of all IWHs in the dataset. In the Rottweiler dataset there were 31 individuals (19 females and 12 males) diagnosed with osteosarcoma, representing 3.1% of all DNA sampled individuals. There were no differences between the sexes in the proportion of individuals affected by osteosarcoma in IWHs – χ^2 test result = 0.065 (p = 0.80), or Rottweilers – χ^2 test result = 0.17 (p = 0.68). Significant differences were identified in the age at osteosarcoma diagnosis between males and females in in both breeds. Mean age at osteosarcoma diagnosis for male IWHs was 5.34 years, whereas the mean age of osteosarcoma diagnosis for female IWHs was 7.65 years (t = 3.04, p = 0.0069). A similar difference in the age at diagnosis was observed in Rottweilers, the mean age at diagnosis for males was 7.00 years while the mean age for diagnosis in females was 8.50 years (t = 2.34, p = 0.027).

Of IWH males, 80% that developed osteosarcoma had done so before the age of 6.5 years, while 80% of IWH females were diagnosed with osteosarcoma by the age of 9 years. A greater number of Rottweilers than IWH live longer, therefore a 90% threshold was used to determine the age restrictions in this

breed. Of male Rottweilers 90% diagnosed with osteosarcoma were diagnosed before the age of 9 years, whereas 90% of females were diagnosed before the age of 10 years. Using these age restrictions there were 23 male IWHs unaffected by osteosarcoma over 6.5 years and 6 female IWHs unaffected by osteosarcoma over 9 years. There were 51 male Rottweilers unaffected by osteosarcoma over the age of 9 years and 53 female Rottweilers unaffected by osteosarcoma over 10 years old.

Previous work identified a relationship between DCM and AF in IWHs (Chapter 4). Also identified was a difference in the age of onset of DCM/AF between males and females, but no difference in the proportion of individuals affected (Chapter 4). Utilising this information the age restrictions that 80% of individuals were affected by DCM/AF had been determined to be 6.5 years for males and 8.5 years for females (Chapter 4). There were 72 IWHs diagnosed with DCM or AF, 45 females and 27 males. There were 9 female IWHs unaffected by DCM or AF over the age of 8.5 years, and 14 male IWHs unaffected by DCM or AF over the age of 6.5 years.

Across all individuals genotyped at both *AR1* and *AR2*, the total length of the AR polyglutamine tract ranged from 33 to 35 repeats. The length of the *AR1* polyglutamine tract ranged from 10 to 12 repeats and the length of the *AR2* polyglutamine tract ranged from 23 to 25 repeats (Figure 6.1). *NCOA3* had a range of 15 to 16 repeats within the polyglutamine tract across all genotyped individuals (Figure 6.1). There were no significant differences in mean *AR* polyglutamine repeat length between IWHs with a diagnosis of DCM/AF and IWHs included in the unaffected by DCM/AF group ($t = 1.13$, $p = 0.27$). There were also no significant differences in mean *AR* polyglutamine repeat length between IWHs with a diagnosis of osteosarcoma and IWHs included in the unaffected by osteosarcoma group ($t = 2.04$, $p = 0.053$), or Rottweilers with a diagnosis of osteosarcoma and Rottweilers included in the unaffected by osteosarcoma group ($t = 1.00$, $p = 0.33$). When individuals were split by sex there remained no significant differences between groups IWH DCM/AF:

ANOVA – F = 0.54, p = 0.66,

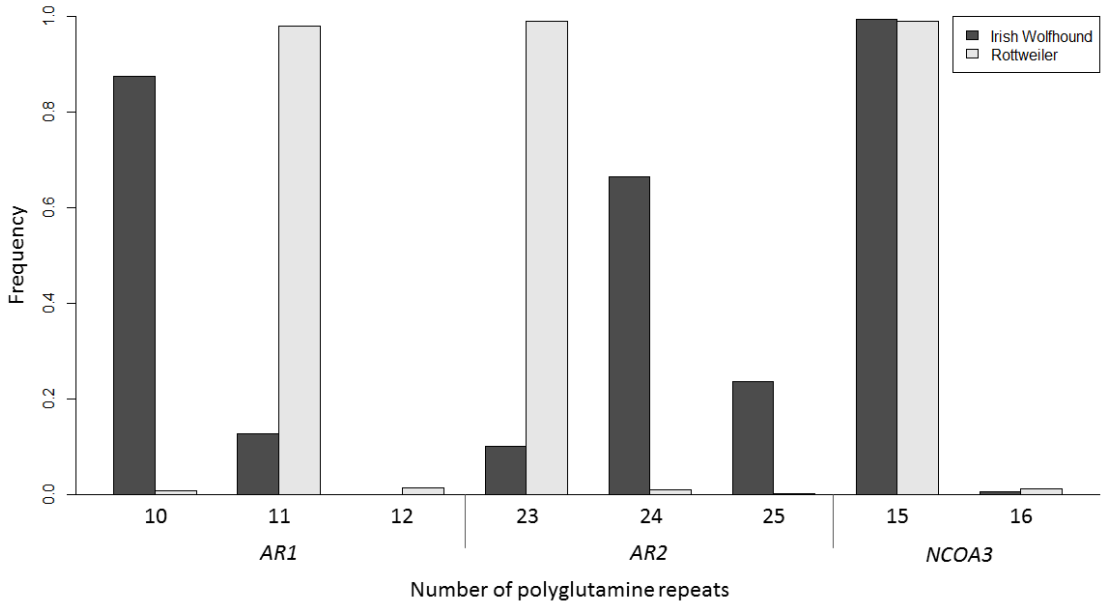


Figure 6.1. Polyglutamine repeat allele frequencies for *AR1, AR2,* and *NCOA3* for IWHs and Rottweilers.

Table 6.2; IWH osteosarcoma: ANOVA – F = 1.28, p = 0.30, Table 6.3; Rottweiler osteosarcoma: ANOVA – F = 1.88, p = 0.14,

Table 6.4. Similar results were obtained when *AR1* and *AR2* were considered separately. There were no significant differences in *AR1* or *AR2* length between IWHs with a diagnosis of DCM/AF and IWHs included in the unaffected by DCM/AF group (*AR1* t = 0.46, p = 0.65; *AR2* t = 2.05, p = 0.052). When individuals were split by sex there remained no significant differences between groups ANOVA – *AR1* F = 0.94, p = 0.42, Table 6.5; *AR2* F = 1.40, p = 0.25, Table 6.6. There were also no significant differences in *AR1* or *AR2* length between individuals with osteosarcoma and without osteosarcoma in IWHs (*AR1* t = 1.02, p = 0.33; *AR2* t = 1.83, p = 0.08) or Rottweilers (*AR1* t = 1.00, p = 0.33; *AR2* t = 1.00, p = 0.32). The lack of significant differences remained when split by sex ANOVA – IWH: *AR1* F = 0.80, p = 0.50, Table 6.7; *AR2* F = 1.04, p = 0.39, Table 6.8; Rottweiler: *AR1* F = 1.88, p = 0.14, Table 6.9; *AR2* F = 0.55, p = 0.65, Table 6.10.

There were no significant differences in mean *NCOA3* polyglutamine repeat length between IWHs with a diagnosis of DCM/AF and IWHs included in the unaffected by DCM/AF group, or IWHs with a diagnosis of osteosarcoma and IWHs included in the unaffected by osteosarcoma group, as all individuals in had 15 repeats in their polyglutamine tract. There were two IWH individuals in the study that had 16 repeats, these individuals were unaffected by AF, DCM, and osteosarcoma, but had not yet reached the age restrictions to be included in the unaffected groups. There was similarly little variation in the *NCOA3* polyglutamine repeat length in Rottweilers with two individuals in the unaffected by osteosarcoma group having mean repeat length of 15.5, the resulting t-test was not significant (t = 1.42, p = 0.16). Due to lack of variation in the length of the *NCOA3* polyglutamine tract individuals were not split by sex.

There were significant differences in *AR1* and *AR2* polyglutamine repeat frequencies between the two breeds, *AR1* χ^2 test result = 1707 (p = <0.00001) and *AR2* χ^2 test result = 1687 (p = <0.00001). IWHs had fewer repeats at *AR1*, but more repeats at *AR2*, compared to Rottweilers (Figure 6.1). t-test of the mean repeat length of *AR* is significantly different with IWHs having a mean of 34.24 repeats and Rottweilers having a mean of 34.02 repeats (t = 10.62, p =

<0.0001). There was no significant difference between breeds in the *NCOA3* polyglutamine repeat frequencies, χ^2 test result = 1.79, (p = 0.18).

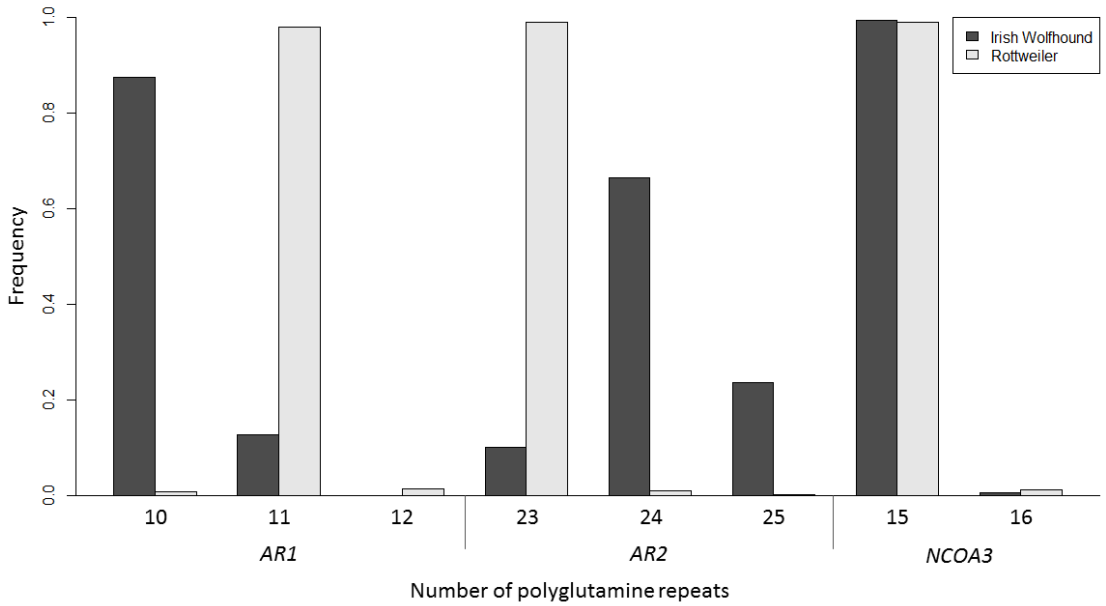


Figure 6.1. Polyglutamine repeat allele frequencies for *AR1*, *AR2*, and *NCOA3* for IWHs and Rottweilers.

Table 6.2. Mean and standard error of the mean (SEM) of the number of *AR* repeats IWHs have in each DCM/AF vs not DCM/AF diagnosis, for each sex.

Group		Number	Mean AR repeat	SEM
Male	DCM/AF	21	34.29	± 0.10
	not DCM/AF over 6.5 years	10	34.40	± 0.16
Female	DCM/AF	38	34.24	± 0.06
	not DCM/AF over 8.5 years	9	34.39	± 0.16

Table 6.3. Mean and standard error of the mean (SEM) of the number of *AR* repeats IWHs have in each osteosarcoma/not osteosarcoma diagnosis, for each sex.

Group		Number	Mean AR repeat	SEM
Male	OSA	4	34.75	± 0.25
	not OSA over 6.5 years	16	34.31	± 0.12
Female	OSA	7	34.57	± 0.13
	not OSA over 9 years	6	34.33	± 0.21

Table 6.4. Mean and standard error of the mean (SEM) of the number of *AR* repeats Rottweilers have in each osteosarcoma/not osteosarcoma diagnosis, for each sex.

Group		Number	Mean AR repeat	SEM
Male	osteosarcoma	12	34	± 0.00
	not osteosarcoma over 9 years	45	33.76	± 0.24
Female	osteosarcoma	18	34.03	± 0.03
	not osteosarcoma over 10 years	43	34	± 0.00

Table 6.5. Mean and standard error of the mean (SEM) of the number of *AR1* repeats IWHs have in each DCM/AF vs not DCM/AF diagnosis, for each sex.

Group		Number	Mean AR1 repeat	SEM
Male	DCM/AF	23	10.17	± 0.08
	not DCM/AF over 6.5 years	12	10.08	± 0.08
Female	DCM/AF	40	10.11	± 0.03
	not DCM/AF over 8.5 years	9	10.00	± 0.00

Table 6.6. Mean and standard error of the mean (SEM) of the number of *AR2* repeats IWHs have in each DCM/AF vs not DCM/AF diagnosis, for each sex.

Group		Number	Mean AR2 repeat	SEM
Male	DCM/AF	21	24.10	± 0.14
	not DCM/AF over 6.5 years	10	24.40	± 0.16
Female	DCM/AF	40	24.16	± 0.08
	not DCM/AF over 8.5 years	6	24.50	± 0.22

Table 6.7. Mean and standard error of the mean (SEM) of the number of *AR1* IWHs have in each osteosarcoma/not osteosarcoma diagnosis, for each sex.

Group		Number	Mean AR1 repeat	SEM
Male	osteosarcoma	4	10.25	± 0.25
	not osteosarcoma over 6.5 years	19	10.05	± 0.05
Female	osteosarcoma	8	10.13	± 0.13
	not osteosarcoma over 9 years	13	10.04	± 0.04

Table 6.8. Mean and standard error of the mean (SEM) of the number of *AR2* IWHs have in each osteosarcoma/not osteosarcoma diagnosis, for each sex.

Group		Number	Mean AR2 repeat	SEM
Male	osteosarcoma	4	24.50	± 0.29
	not osteosarcoma over 6.5 years	48	24.31	± 0.12
Female	osteosarcoma	7	24.57	± 0.13
	not osteosarcoma over 9 years	13	24.19	± 0.16

Table 6.9. Mean and standard error of the mean (SEM) of the number of *AR1* Rottweilers have in each osteosarcoma/not osteosarcoma diagnosis, for each sex.

Group		Number	Mean AR1 repeat	SEM
Male	osteosarcoma	12	11.00	± 0.00
	not osteosarcoma over 9 years	16	11.00	± 0.00
Female	osteosarcoma	19	11.03	± 0.03
	not osteosarcoma over 10 years	93	11	± 0.00

Table 6.10. Mean and standard error of the mean (SEM) of the number of *AR2* Rottweilers have in each osteosarcoma/not osteosarcoma diagnosis, for each sex.

Group		Number	Mean AR2 repeat	SEM
Male	osteosarcoma	12	23.00	± 0.00
	not osteosarcoma over 9 years	16	23.00	± 0.00
Female	osteosarcoma	7	23.00	± 0.00
	not osteosarcoma over 10 years	13	22.98	± 0.02

6.5. Discussion

This is the first study to examine the length of the canine *NCOA3* polyglutamine tract. This is also the first evaluation of the *AR* polyglutamine tract in IWHs and Rottweilers. In this study the canine *NCOA3* polyglutamine tract displayed little repeat variation in either IWHs or Rottweilers (Figure 6.1). Most individuals had 15 polyglutamine repeats in both alleles of the *NCOA3* gene, with only one additional repeat in 1% of Rottweiler and 0.5% of IWH *NCOA3* polyglutamine tracts genotyped. The lack of variation in the length of the *NCOA3* polyglutamine tract in these two breeds of dog strongly suggests that variation in the length of the *NCOA3* polyglutamine tract is not a contributing factor in the development of either DCM/AF or osteosarcoma in IWHs, or osteosarcoma in Rottweilers. Due to genotyping two unrelated breeds it is also possible to conclude that there is little variation in the length of the canine *NCOA3* polyglutamine tract. The length of the canine *AR* polyglutamine tract has previously been associated with aggression in male Japanese Akita Inus and prostate cancer in a variety of breeds (Konno et al., 2011, Lai et al., 2008). In the current study there was no association with the length of the canine *AR* polyglutamine tract and DCM/AF or osteosarcoma in either IWHs or

Rottweilers. These results do not rule out an association of *AR* or *NCOA3* with either disease, as only the *AR*-polyglutamine tracts were examined.

There is some evidence that *AR* and/or androgens could play a role in the development and progression of human osteosarcoma. AR protein expression is elevated in higher clinical stage and histological grade tumours than lower clinical stage and histological grade tumours (Wagle et al., 2015). This was in combination with a co-activator (CCAR2) but Wagle *et al.* (2015) did not examine either gene for variation so it is not possible to know if this difference in expression is due to genetic variation in either the *AR* or *CCAR2* genes. As described elsewhere in this thesis, RNAseq did not identify differential expression of the *AR*, *NCOA3 or CCAR2* in canine osteosarcoma relative to unaffected bone tissue. It is possible that there is variation in canine osteosarcoma between tumour and non-tumour tissue in the expression of protein, but not RNA, however it is not possible to determine this based on this current study or previous analysis (Ghazalpour et al., 2011, Gry et al., 2009). Further work would be required to establish if there is a difference in protein expression of NCOA3 between canine osteosarcoma tumour and non-tumour tissue.

Human DCM can be caused by mutations in the *LMNA* gene and it has been shown that AR accumulates in the nuclei of *LMNA* mutation affected individuals (Arimura et al., 2013). In this case it has been shown that the accumulation of AR and the subsequent response to testosterone is associated with DCM progression (Arimura et al., 2013). Due to males producing greater amounts of testosterone than females this may explain the difference between sexes in disease incidence and progression in *LMNA* mutation affected individuals (Arimura et al., 2013). Conversely it has been shown that in AR knockout mice the incidence of AF increases (Tsai et al., 2013). AF has been associated with the development of DCM in IWHs (Chapter 4), but it is not associated with the development of DCM in people (Unverferth et al., 1984, Wasywich et al., 2010). The presence of AF in people with DCM and heart failure has, however, been shown to reduce survival (Unverferth et al., 1984, Wasywich et al., 2010). It is not clear if or how aberrant expression of AR could affect the development of

canine DCM and AF, therefore additional work is warranted. In the current study the expression of the androgen receptor gene and protein were not assessed so it is not possible to establish if this could be an alternative possibility for the mode of action of *AR* and androgens in the development of canine DCM and AF. Future work examining the expression of the androgen receptor gene and protein in canine DCM/AF and non-DCM/AF cardiac tissue could be useful in establishing mechanisms of action for the different age at onset of canine DCM/AF between the sexes.

The frequency of the number of repeats (allele frequency) was previously established in a range of breeds, but the length of the androgen receptor polyglutamine tract has not previously been assessed in any context in IWHs or Rottweilers (Maejima et al., 2005). Both breeds assessed in the current study have similar repeat length distributions to each other (Figure 6.1) and to those previously reported by Maejima *et al.* (2005). However it is interesting that IWHs and Rottweilers have significantly different allele frequencies at both *AR1* and *AR2* (Figure 6.1), however Maejima *et al.* (2005) reported a range of allele frequencies across breeds. This difference in allele frequencies can be attributed to different population histories, with founder effects, inbreeding, and genetic hitchhiking all potentially affecting the allele frequencies (Fu, 1997, Slatkin, 1995, Wright, 1949).

6.6. Conclusions

In conclusion variation in the polyglutamine repeat tracts of the *AR* and *NCOA3* genes is not associated with the development of canine osteosarcoma or DCM/AF in IWHs and Rottweilers. This does not preclude variation elsewhere in either or both genes to have functional importance and association with canine osteosarcoma or DCM/AF. Further work is required to establish if the response to androgens is indeed the underlying cause of the sex differences in the development of canine osteosarcoma and DCM/AF, or if there is an alternative cause of the sex differences.

Chapter 7 Fatty acid profiles and canine heart disease

7.1. Abstract

Cardiovascular disease is a leading cause of death from non-communicable causes in both the human and canine populations. Heart failure can be the result of many cardiac disease processes including both dilated cardiomyopathy and valve disease. Dilated cardiomyopathy tends to affect younger people, while valve disease is more common in older people. Both dilated cardiomyopathy and valve disease occur at high incidences in the human and canine populations. Heart failure is associated with markers of inflammation and important aspects of the inflammatory response are fatty acid-derived eicosanoids. Fatty acid profiles have been shown to be different in a number of human and canine diseases. In this study the fatty acid profiles of dogs with dilated cardiomyopathy, valve disease, and other non-cardiac diseases were examined to establish whether there could be a difference in the inflammatory response between diagnoses. It was found that individuals with valve disease had different fatty acid profiles from individuals with dilated cardiomyopathy and non-cardiac diseases. In particular individuals with valve disease had a greater contribution of fatty acids from the palmitic acid pathway than individuals with dilated cardiomyopathy and non-cardiac diseases. In contrast individuals with dilated cardiomyopathy and non-cardiac diseases had a greater contribution of fatty acids from the linoleic acid pathway. Further work is warranted to elucidate implications of these differences and establish how these profiles compare to healthy individuals, but drugs modifying fatty acid synthesis and metabolism could be therapeutic targets in cardiac disease.

7.2. Introduction

Cardiovascular disease is the leading cause of death from non-communicable disease in the human population, with deaths attributable to cardiovascular disease predicted to rise to over 20 million per year by 2030 (Mathers and Loncar, 2006). Although there are differences between species, animal models of cardiac disease can lead to advances in human medicine (Cheng et al., 2016, Houser et al., 2012). The pet dog population may represent comparative

models of human disease because diseases, including cardiovascular disease, progress under clinically relevant timescales and dogs are subject to similar environmental effects as humans (Ostrander et al., 2000). In the general canine population, cardiac disease incidence has been reported to be among the top 5 causes of causes of death, though certain breeds are more commonly affected (Bonnett et al., 1997, Inoue et al., 2015, O'Neill et al., 2013). The leading specific causes of canine cardiac death are dilated cardiomyopathy (DCM) and myxomatous degeneration/degenerative mitral valve disease (Egenvall et al., 2006). In humans, DCM is the most common form of cardiomyopathy in children, whereas the incidence of valve disease is increasing due to the aging population (Mozaffarian et al., 2015). Both DCM and valve disease can lead to heart failure (Kupari et al., 1997, Olson, 1998).

Many diseases, including heart disease, involve inflammatory responses in either the initiation of the disease process or as a reaction to disease; although inflammation itself is not always detrimental, for instance it is an important aspect of wound repair (Danesh et al., 2000, Gurtner et al., 2008, Harris, 2009, Mohler et al., 2001). Heart failure is associated with increases in markers of inflammation which could be either the cause of disease or elevated in response to heart failure (Aukrust et al., 1998, Leyva et al., 1998, Pankuweit et al., 2013).

Important contributors to the inflammatory response are fatty acid-derived eicosanoids (Bagga et al., 2003, Harris, 2009, Vane, 1971). Fatty acids consist of a carboxylic acid with a hydrocarbon chain tail (Fahy et al., 2005). The length of the hydrocarbon chain varies between fatty acids, as does the presence or absence of double bonds between the carbon atoms and their location (Fahy et al., 2005). In addition to being the precursors for eicosanoids and other metabolites, fatty acids are major substrates for energy production, and are essential components of the cell membrane (Fahy et al., 2005, Kröger et al., 2014, Wolters et al., 2014).

In mammals, fatty acids are obtained from the diet and can be synthesised into other fatty acids by elongation and desaturation enzymes as depicted in Figures

7.1 and 7.2 (Farvid et al., 2014, Kanehisa et al., 2016, Meyer et al., 2003, Vessby et al., 2013, Wang et al., 2005b). There are two major groups of fatty acids: ω-6 fatty acids are primarily obtained from fats and oils, and ω-3 fatty acids which are obtained primarily from fish and seafood products (Meyer et al., 2003). Synthesis of fatty acids occurs along specific pathways, although mammals are not naturally able to convert between the two major pathways and therefore must obtain precursors of both ω-6 and ω-3 fatty acids from the diet (Kang et al., 2004).

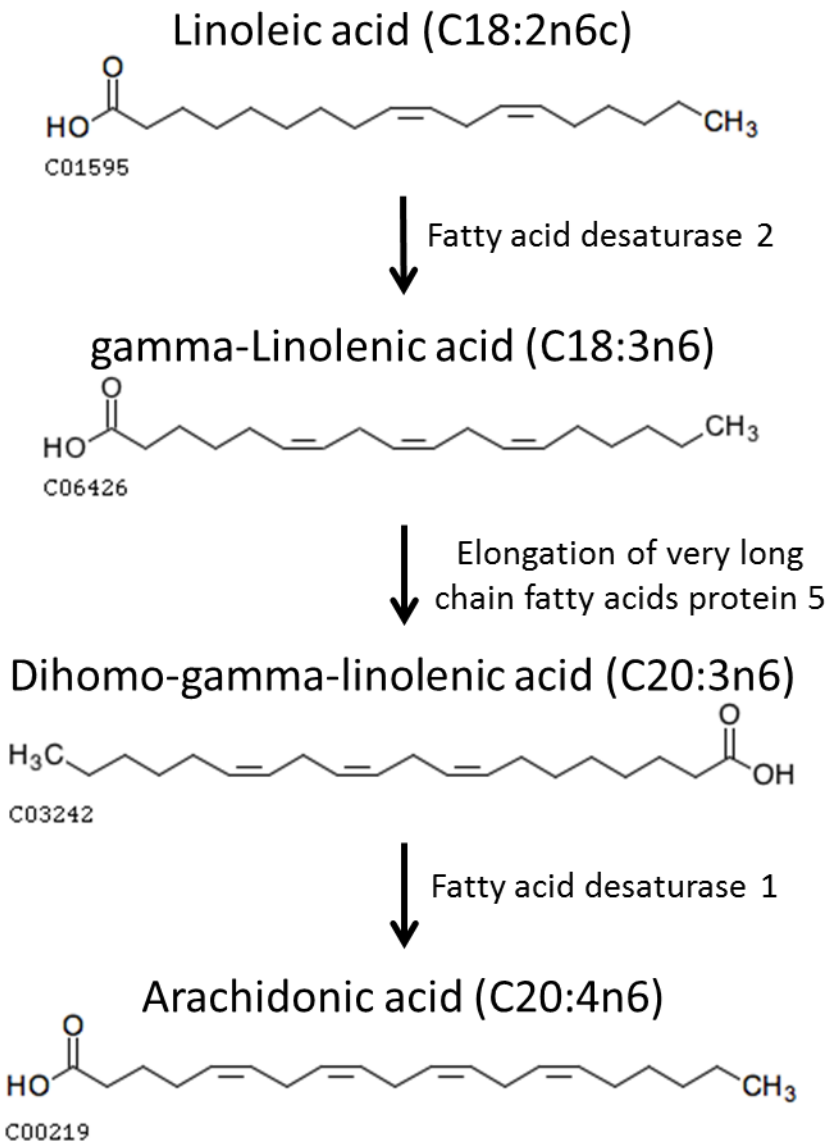


Figure 7.1. Schematic of linoleic and arachidonic acid biosynthetic pathway adapted from KEGG pathway maps (Kanehisa et al., 2016).

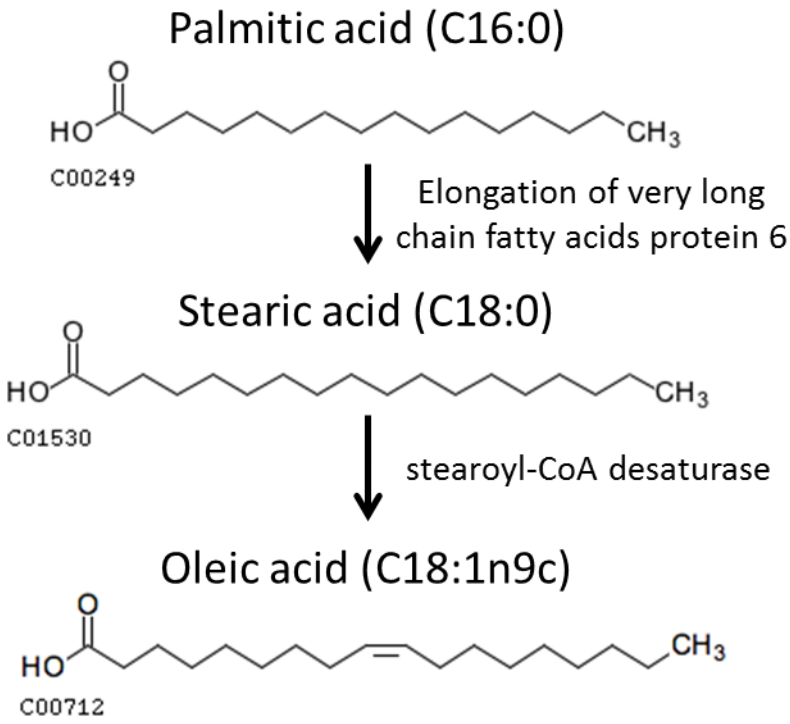


Figure 7.2. Schematic of palmitic and oleic acid biosynthesis pathway adapted from KEGG pathway maps (Kanehisa et al., 2016).

The ω-6 fatty acid arachidonic acid is a precursor of the most important pro-inflammatory eicosanoids, while the ω-3 fatty acid derivatives, eicosapentaenoic acid and docosahexaenoic acid metabolites are considered less inflammatory (Bagga et al., 2003). In response to pro-inflammatory stimuli including immune-mediated processes and cytokines, arachidonic acid is released from cell membranes by phospholipase A_2 enzymes (Bonventre et al., 1997, Burch et al., 1986, Lee et al., 2010, Xu et al., 2003). This free arachidonic acid is then available for conversion to eicosanoids by cyclooxygenase, lipoxygenase and cytochrome P450 enzymes (McAdam et al., 1999, Morrison and Pascoe, 1981, Oliw et al., 1981, Palmer et al., 1980). These enzymes are also responsible for the conversion of other fatty acids to their metabolites resulting in rate limiting competition (Bagga et al., 2003). There is some evidence that by increasing the amount of ω-3 fatty acids available to the cyclooxygenase, lipoxygenase, and cytochrome P450 enzymes fewer arachidonic acid derived eicosanoids are produced which could reduce inflammation (Bagga et al., 2003, Todoric et al., 2006). Consistent with this, fatty acid profiles have been shown to be altered in blood and tissues in

individuals with a range of conditions compared to unaffected individuals in both humans and dogs. These conditions include heart disease, skin disease, and cancer (Azordegan et al., 2012, Baylin and Campos, 2004, Rupp et al., 2010, Taugbøl et al., 1998). However it remains unknown whether this difference in fatty acid profiles between unaffected, control individuals and those with disease is a contributing factor to the initiation of disease or as a consequence of disease. Indeed while it may be expected that fatty acid profiles differ between healthy individuals and individuals with diseases with an inflammatory component, there may also be differences in the fatty acid profiles between diseases. In this study the hypothesis that the fatty acid profiles in the blood of canine patients differs between disease states, namely non-cardiac disease, DCM and valve disease, is tested.

7.3. Methods

7.3.1. Samples

This study was approved by the University of Nottingham ethics committee in compliance with the Home Office regulations and the Veterinary Surgeons Act. Informed consent was obtained from all dog owners involved in this research. Remnants of whole blood samples taken from canine patients for veterinary diagnostic purposes were obtained and stored at -80°C (samples collected by cardiac specialist Professor Malcolm Cobb). Fatty acids were extracted from patients with a variety of diagnoses. These formed three patient groups: Group 1 – 10 dogs with DCM, Group 2 – 18 dogs with other heart diseases, and Group 3 – 4 control dogs which had not been diagnosed with any form of cardiac disease. The samples were age, sex and breed matched where possible. Details of the dogs are shown in Table 7.1

Table 7.1 Diagnosis, age at diagnosis, sex and breed of all dogs used for fatty acid analysis.

Diagnosis	Age at diagnosis	Sex	Breed
DCM	Not available	M	Cocker Spaniel
DCM	6years 10months	FN	Great Dane
DCM	4years 3months	MN	Weimeraner
DCM	4years 10months	MN	Cocker Spaniel
DCM	7years 8 months	MN	Doberman
DCM	7years 6months	M	Boxer
DCM	11years 8months	MN	English Springer Spaniel
DCM	5years 3months	MN	Doberman
DCM	6years 3months	MN	Dogue de Bordeaux
DCM/end stage valve disease	6years 11months	ME	Deerhound
MVD and CHF	10years 3months	FN	Cavalier King Charles Spaniel
MVD	6years	M	Cavalier King Charles Spaniel
MVD	5years 4months	M	Cavalier King Charles Spaniel
MVD	15 years 6 months	FN	Cross breed
MVD	Not available	MN	Yorkshire Terrier
MVD	10years	ME	Cavalier King Charles Spaniel
MVD	7years 6months	M	Cavalier King Charles Spaniel
MVD	Not available	MN	Cavalier King Charles Spaniel
MVD	Not available	FN	Yorkshire Terrier
MVD	7years 5months	F	Cavalier King Charles Spaniel
MVD	8years 5months	ME	Bull Terrier
MVD	12years 6months	MN	Lhasa Apso
MVD and CHF	8years 7months	MN	English Springer Spaniel
MVD and CHF	Not available	M	Yorkshire Terrier
MVD and CHF	12years 4months	MN	Yorkshire Terrier
SAS	Not available	M	Boxer
TVD and AF	Not available	ME	Mastiff
valve disease and lung mass	8years	MN	Weimeraner
no cardiac diagnosis, aural haematoma	4years 8months	ME	Labrador
no cardiac diagnosis, RACL, skin disease	5years 2 months	MN	English Springer Spaniel
no cardiac diagnosis, myelofibrosis	7year 8months	FN	Boxer
no cardiac diagnosis, bone neoplasia	10years 5 months	MN	Collie

Diagnosis abbreviations: DCM – Dilated Cardiomyopathy, MVD – Mitral Valve Disease, TVD – Tricuspid Valve Disease, SAS – Subvalvular Aortic Stenosis, AF – Atrial Fibrillation, CHF – Congestive Heart Failure, RACL – Ruptured Anterior Cruciate Ligament. Sex is indicated by M for male and F for female with N indicating that the individual was neutered and E that it was entire, where N or E is absent this data was not available. Age at disease diagnosis is stated where this information was available.

7.3.2. Fatty acid extraction, methylation, and gas chromatography

Fatty acids were extracted from 200µL whole blood using a modified Folch method (Folch et al., 1957). 750µL of cold chloroform:methanol (1:2) (Chloroform: Sigma-Aldrich, USA, Methanol: Thermo Fisher Scientific, USA) was vortexed for 1 minute with the blood sample in a borosilicate glass tube (KIMAX™, Kimble™, USA). To this 250µL cold chloroform and 250µl H_2O were added prior to vortexing for an additional 1 minute. Samples were then centrifuged at 1,100x g for 15 minutes at 15°C. The organic chloroform phase containing the fatty acids was transferred to a clean borosilicate glass tube and dried under a nitrogen stream. Samples were re-dissolved in 400µL hexane (Thermo Fisher Scientific, USA) prior to trans-esterification by the method of Christie (1982) modified by Chouinard *et al.* (1999), by vortexing 8µL of methyl acetate (Sigma-Aldrich, USA) and 8µL methylation reagent with samples. The methylation reagent consisted of 0.45ml of 30% sodium methoxide (Sigma-Aldrich, USA) and 2.05ml of methanol (Thermo Fisher Scientific, USA). This was allowed to react at room temperature for 10 minutes and then 12µL of termination reagent consisting of 0.1g of oxalic acid (Sigma-Aldrich, USA) in 3mL of diethyl ether (Sigma-Aldrich, USA) was added and briefly vortexed. 200mg of calcium chloride (Thermo Fisher Scientific, USA) was added to each sample, vortexed and incubated at room temperature for 1 hour. The samples were centrifuged for 1,100x g for 15 minutes at 15°C and the supernatant transferred to a gas chromatography vial for use directly in gas chromatography. Fatty acid isolation protocols and guidance were generously provided by Dr. Alison Mostyn, University of Nottingham and is gratefully acknowledged.

The fatty acid methyl esters were injected into a gas chromatograph (6890 Gas Chromatograph, Agilent, USA) by a split injection (ratio 50:1) with hydrogen used as the carrier gas. Separation of the fatty acid methyl esters was performed using a Varian CP-Sil 88 capillary column (Crawford Scientific Ltd, UK). The oven temperature was programmed to increase from 59°C to 100°C at a rate of 8°C per minute, it was then increased to 170°C at a rate of 6°C per minute and held for 10 minutes. Following this the oven temperature was then increased to 240°C at a rate of 3°C per minute and held for 10 minutes. The

temperature of the injector and detector were set at 255°C and 250°C respectively. The fatty acid methyl esters were identified by comparing the retention times with a fatty acid methyl esters standard mixture (Sigma-Aldrich, USA). The area percentage in moles of each fatty acid were used for the statistical analysis. The fatty acids analysed included saturated fatty acids: C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0, C24:0; monounsaturated fatty acids: C14:1, C15:1, C16:1, C17:1, C20:1, C24:1; polyunsaturated fatty acids: omega (n)-3: C18:3n3, C20:3n3, C20:5n3, C22:6n3, omega (n)-6: C18:2n6t, C18:2n6c, C18:3n6, C20:2n6, C20:3n6, C20:4n6, omega (n)-9: C18:1n9t, C18:1n9C, C22:1n9. The gas chromatography machine was run as a service by Dr Dongfang Li, School of Biosciences, University of Nottingham.

7.3.3. Fatty acid statistical analysis

The gas chromatography method quantifies an area value for each fatty acid. The relative abundance of each fatty acid in a sample was obtained by calculating the percentage each fatty acid contributed to the overall profile. To establish which individuals clustered based on their fatty acid profiles, unsupervised hierarchical clustering analysis was performed using Cluster3.0 (de Hoon et al., 2004). The data was filtered so that there were at least four individuals with a measurement ≥0.1% for a gene (fatty acid). Fatty acids and arrays (samples) were mean-centred and hierarchical clustering of fatty acids and samples performed using average linkage. Fishers exact tests were performed using R statistical software (RCoreTeam, 2015) to establish whether there are differences in the number of individuals with DCM, valve disease, and no cardiac diagnosis between the two large clusters identified. This was based on the counts of individuals with each diagnosis type in each cluster. There were smaller clusters identified, but the sample size was not large enough to further test for differences between groups.

ANOVA tests were performed to establish whether there were differences between the percentages of any of the fatty acids between each of the groups and a Bonferroni correction was applied to account for multiple tests. Following a significant ANOVA result, post-hoc Tukey tests were performed to establish

which groups were significantly different from each other. ANOVAs and Tukey tests were carried out using R statistical computing (RCoreTeam, 2015).

Where individual significant results were obtained for fatty acids that are related as biosynthetic precursors or products, these were then considered together. The related fatty acids were plotted against each other on scatterplots per group and linear regression of each group was performed using R statistical software (RCoreTeam, 2015) to establish if there were differences in the relationships between fatty acids between groups. The related fatty acids were also combined into a total percentage in each individual and ANOVAs and Tukey tests carried using R statistical software (RCoreTeam, 2015) out to test for differences in the combined percentage of these fatty acids.

There were significant fatty acids that were related to each other as biosynthetic precursors or products from two separate fatty acid pathways. To test whether there was a relationship between pathway fatty acid percentages, the percentages of the significant fatty acids within each pathway were combined and plotted on a scatter plot with associated regression lines using R statistical software (RCoreTeam, 2015). The percentages from both pathways were combined into a single overall percentage for each individual for determination of ANOVA.

7.4. Results

7.4.1. Cluster analysis

Filtering the fatty acids so that there were at least four individuals with a measurement ≥0.1% removed 16 fatty acids. Thus, 20 fatty acids remained in the dataset, specifically the saturated fatty acids: C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0 and the monounsaturated fatty acids: C16:1, C17:1, C20:1, C24:1; polyunsaturated fatty acids: omega (n)-3: C18:3n3, C20:5n3, C22:6n3, omega (n)-6: C18:2n6c, C20:2n6 C20:3n6, C20:4n6, omega (n)-9: C18:1n9t, C18:1n9c. Two main groups were identified, Figure 7.3. These groups contained a mixture of breeds, sex, neuter status, and diagnoses. Fisher's exact tests showed that there was no difference between the groups in the number of individuals of each sex, neuter status, or breed. There were

significant differences in the number of individuals of each diagnosis between the groups though (Fishers exact test p = 0.0014). All the patients with no cardiac diagnosis were in one group alongside 9 out of 11 of the DCM patients, while 10 out of 13 of the MVD and all other valve disease patients presented in a second cluster.

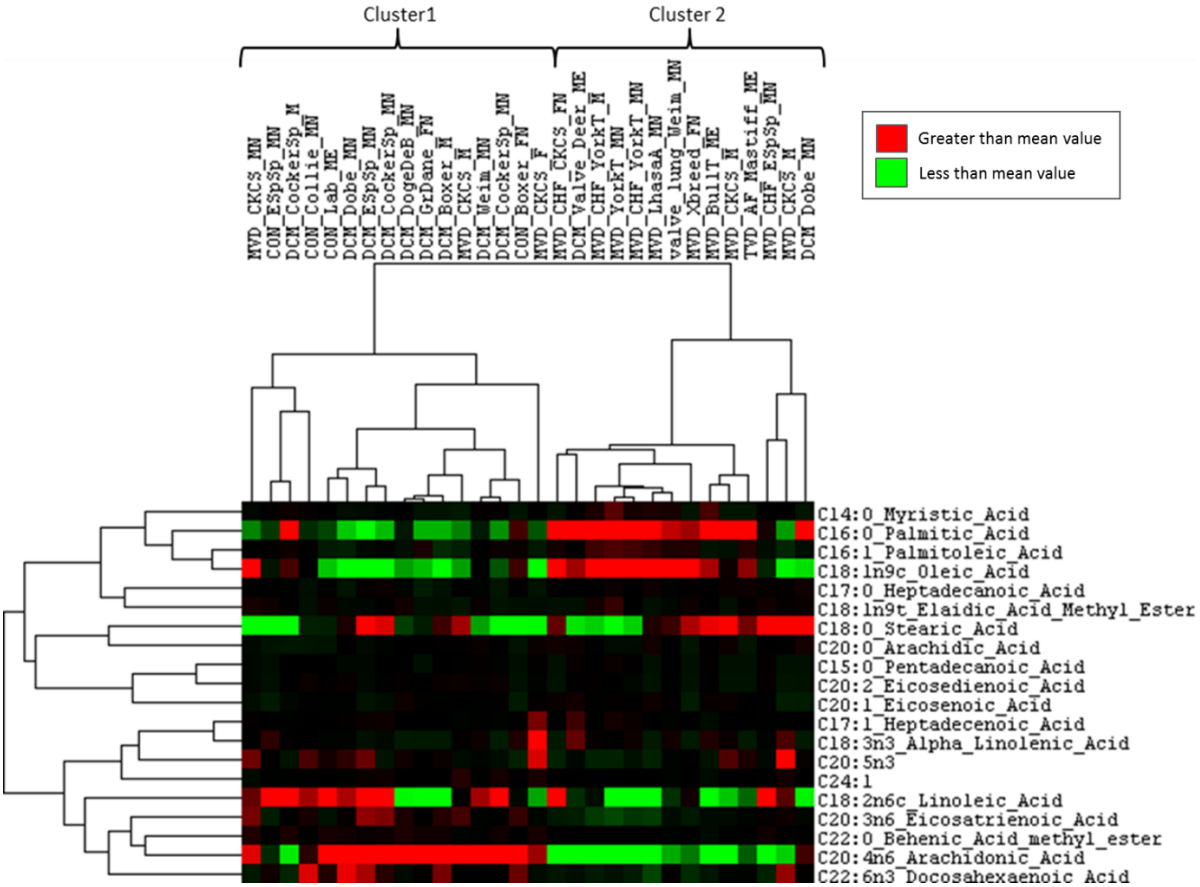


Figure 7.3. Heat map of fatty acid cluster analysis results. Each sample is represented by a single column, sample names include diagnosis, breed and sex. Red indicates an individual has greater than the mean value for that fatty acid, green indicates less than the mean value, the brighter the colour the greater the difference between the individual and the mean value. Abbreviations: MVD – mitral valve disease, CON – non-heart disease patient, DCM – dilated cardiomyopathy, valve – non-specific valve disease, TVD – tricuspid valve disease, CHF – congestive heart failure, AF – atrial fibrilation. CKCS – Cavalier King Charles Spaniel, ESpSp – English Springer Spaniel, CockerSp – Cocker Spaniel, Lab – Labrador, Dobe – Doberman, DogeDeB – Dogue de Bordeaux, GrDane – Great Dane, Weim – Weimaraner, Deer – Deerhound, YorkT – Yorkshire Terrier, LhasaA – Lhasa Apso, Xbreed – Cross Breed, BullT – Bull Terrier.

7.4.2. ANOVA analysis of fatty acids in dilated cardiomyopathy, mitral valve disease, and non-cardiac patients

The unsupervised hierarchical cluster analysis showed that apart from three individuals with MVD, all types of valve disease resulted in similar fatty acid profiles. Based on this clustering, all individuals with any valve disease diagnoses were grouped together for analysis by ANOVA of individual fatty acids. Following the Bonferroni correction only one fatty acid showed a significant difference between the two groups, Arachidonic acid (C20:4n6) ($F = 10.2$, $p = 0.0005$). The post-hoc Tukey test showed that the difference in Arachidonic acid percentage was between the valve disease group and the DCM group (difference = -8.13, $p = 0.0004$). The mean Arachidonic acid percentage of the valve disease group was lower than the means of both the DCM group (significant) and no cardiac diagnosis group (difference = -6.04, $p = 0.0722$, not significant). There were significant ($p \leq 0.05$) differences between groups identified by ANOVA for six additional fatty acids, though these lost significance following Bonferroni correction. These are shown in Table 7.2

Table 7.2. ANOVA results and mean percentage of each fatty acid in each group.

Fatty acid	F	p	Mean control %	Mean DCM %	Mean valve %
C20:4n6_Arachidonic_Acid	14.42	**0.000055**	18.16	20.34	12.12
C16:0_Palmitic_Acid	6.80	0.0041	17.73	17.21	21.07
C18:1n9c_Oleic_Acid	6.46	0.0051	11.99	10.31	15.91
C17:0_Heptadecanoic_Acid	5.68	0.0087	0.56	0.54	0.69
C20:0_Arachidic_Acid	5.24	0.012	0.00	0.22	0.26
C22:6n3_Docosahexaenoic_Acid	4.58	0.019	1.92	1.33	0.72
C18:2n6c_Linoleic_Acid	3.54	0.043	19.89	17.68	14.89
C20:1_Eicosenoic_Acid	2.74	0.083	0.15	0.24	0.34
C16:1_Palmitoleic_Acid	2.16	0.14	0.83	0.84	1.09
C20:3n6_ Dihomo_gamma_linolenic_acid	2.04	0.15	1.35	1.65	1.20
C18:3n3_Alpha_Linolenic_Acid	2.01	0.15	0.50	0.13	0.58
C14:0_Myristic_Acid	1.75	0.19	0.21	0.16	0.38
C22:0_Behenic_Acid_methyl_ester	1.54	0.23	0.06	0.15	0.06
C17:1_Heptadecenoic_Acid	1.24	0.31	0.00	0.01	0.13
C18:0_Stearic_Acid	1.05	0.36	25.67	28.16	28.40
C18:1n9t_Elaidic_Acid_Methyl_Ester	0.72	0.49	0.16	0.15	0.25
C15:0_Pentadecanoic_Acid	0.61	0.55	0.09	0.11	0.15
C24:1	0.57	0.57	0.00	0.03	0.07
C20:2_Eicosedienoic_Acid	0.44	0.65	0.14	0.19	0.15
C20:5n3	0.34	0.72	0.59	0.54	0.87

P value significant values are highlighted in grey. Values significant following Bonferroni's post-hoc correction are indicated in bold. The control group consists of individuals with no diagnosed cardiac abnormality.

7.4.3. Analysis of biosynthetically related fatty acids

7.4.3.1. Arachidonic acid and linoleic acid

Linoleic acid is a precursor of arachidonic acid (see Figure 7.1 (Kanehisa et al., 2016)), prior to Bonferroni correction the ANOVA for the percentage contribution of linoleic acid gave a significant result (Table 7.2). The scatterplot of arachidonic acid vs linoleic acid with regression lines for each group is shown in Figure 7.4. There was a statistically significant negative relationship between the percentages of arachidonic acid and linoleic acid in the DCM group ($F = 7.896$, $p = 0.023$), a non-significant negative relationship in the control - no cardiac diagnosis group ($F = 2.110$, $p = 0.284$), and a non-significant positive relationship in the valve disease group ($F = 1.563$, $p = 0.233$).

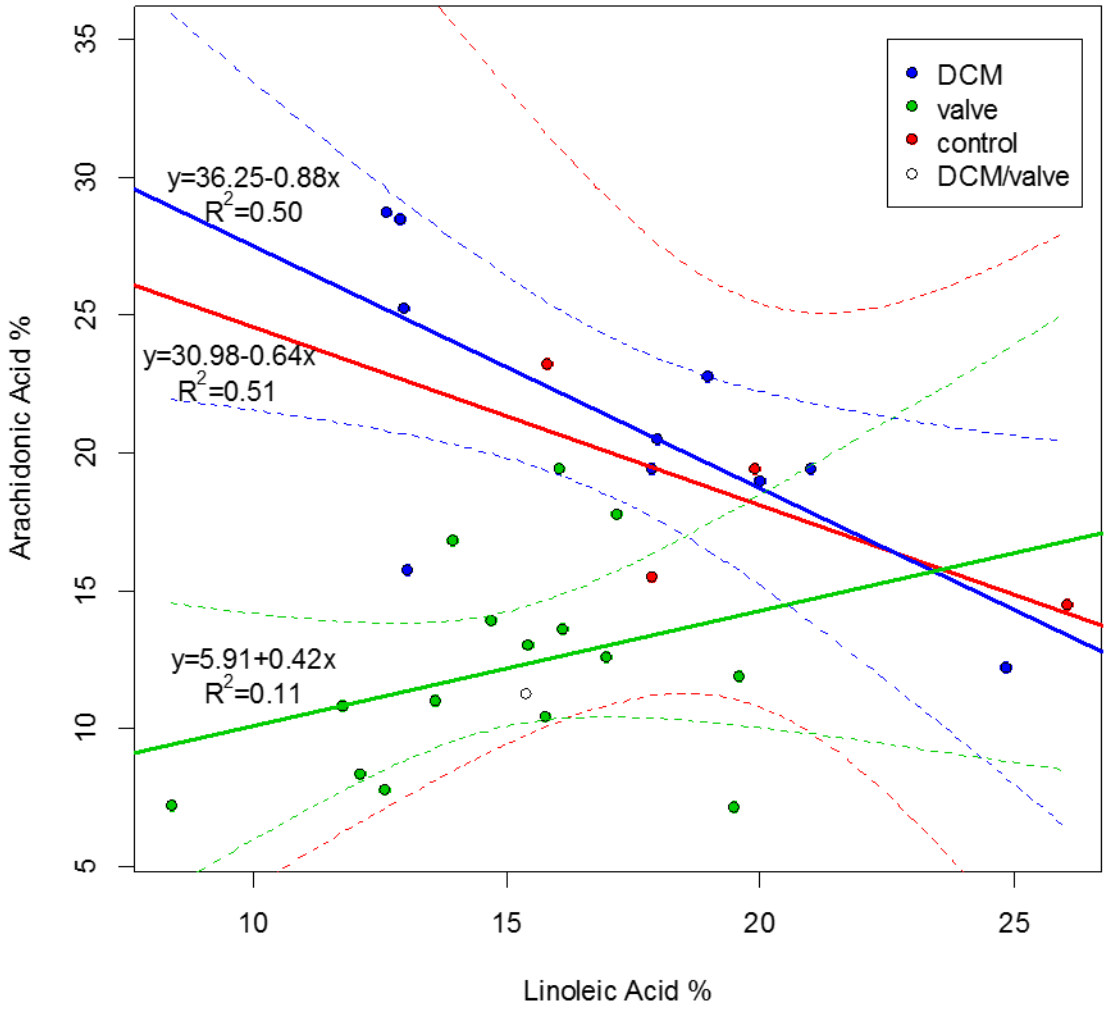


Figure 7.4. Percentage arachidonic acid vs percentage linoleic acid with associated regression lines. 95% confidence intervals are shown as dashed lines. Individuals with a DCM diagnosis are shown in blue, individuals with a valve disease diagnosis are shown in green, and individuals with other diagnoses are shown in red. The non-coloured point indicates the individual which had its diagnosis recorded as "DCM/end stage valve disease".

When the arachidonic acid and linoleic acid percentages were combined, the difference between the valve disease group and the other two groups increased compared to arachidonic acid considered on its own, but the difference between the DCM and no cardiac diagnosis groups reduced. ANOVA ($F = 21.86$, $p = 0.000002$), differences: valve disease and DCM -11.37 ($p = 0.0000055$), valve disease and no cardiac diagnosis -11.07 ($p = 0.00065$), DCM and no cardiac diagnosis 0.30 ($p = 0.99$). Figure 7.5 shows the difference between groups when arachidonic acid and linoleic acid were combined, while Figures 7.6 and

7.7 show the differences between groups for arachidonic acid and linoleic acid individually.

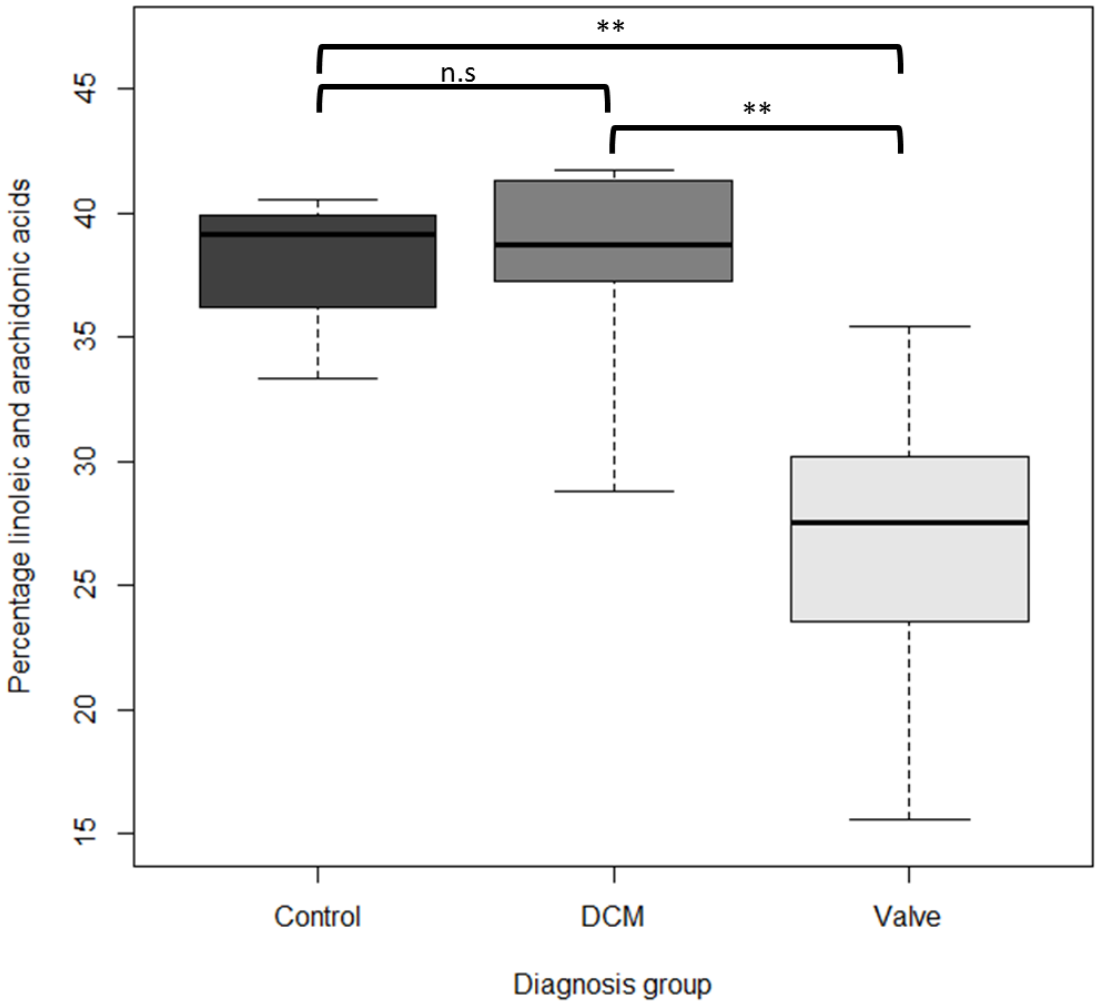


Figure 7.5. Total percentage of fatty acids that were arachidonic acid and linoleic acid for each of the three groups - no cardiac diagnosis, DCM and valve disease. Thick centre line = median, upper and lower edges of boxes = upper and lower quartile ranges respectively, whiskers = full range. ** = $p<0.01$, n.s = not significant.

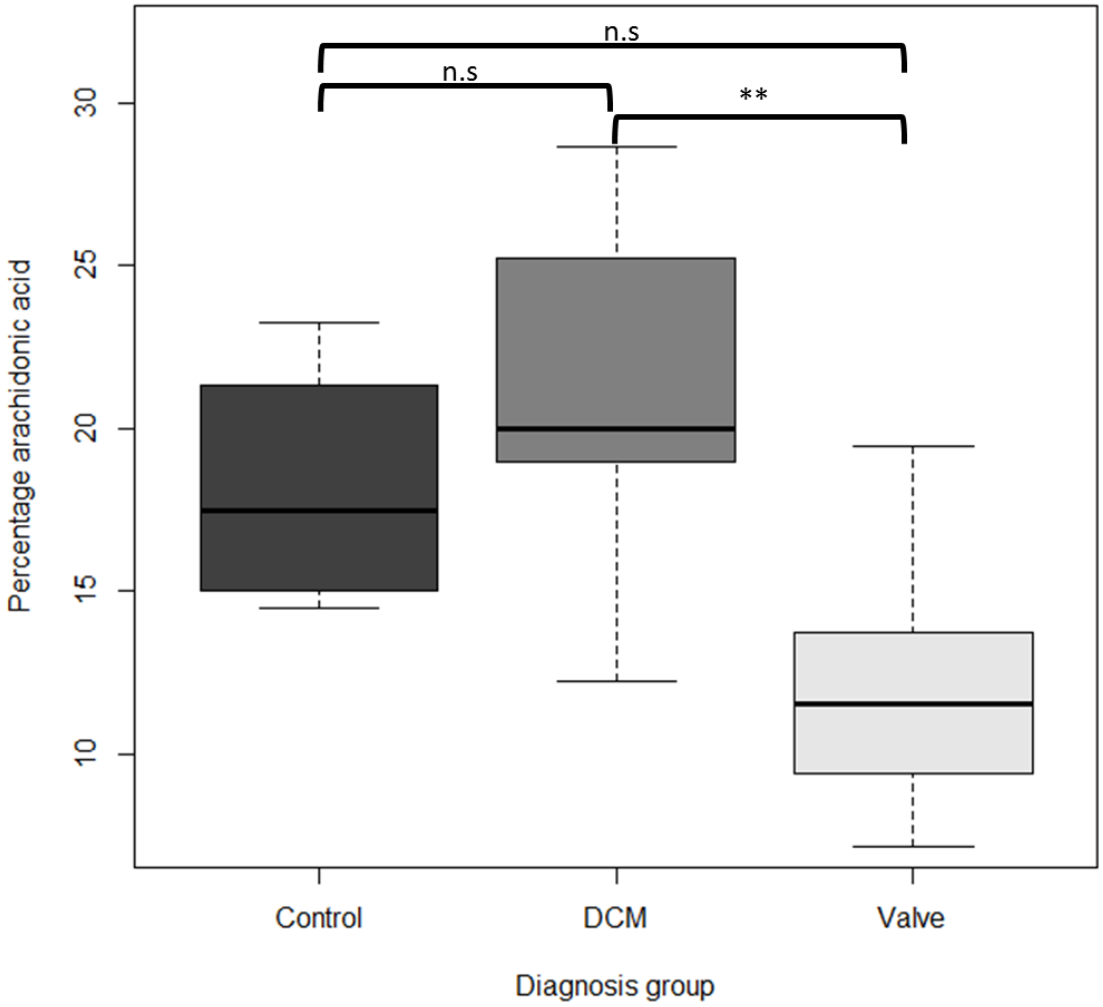


Figure 7.6. Arachidonic acid levels (expressed as a percentage of total fatty acid) in each of the three groups - no cardiac diagnosis, DCM and valve disease. Thick centre line = median, upper and lower edges of boxes = upper and lower quartile ranges respectively, whiskers = full range. ** = $p<0.01$, n.s = not significant.

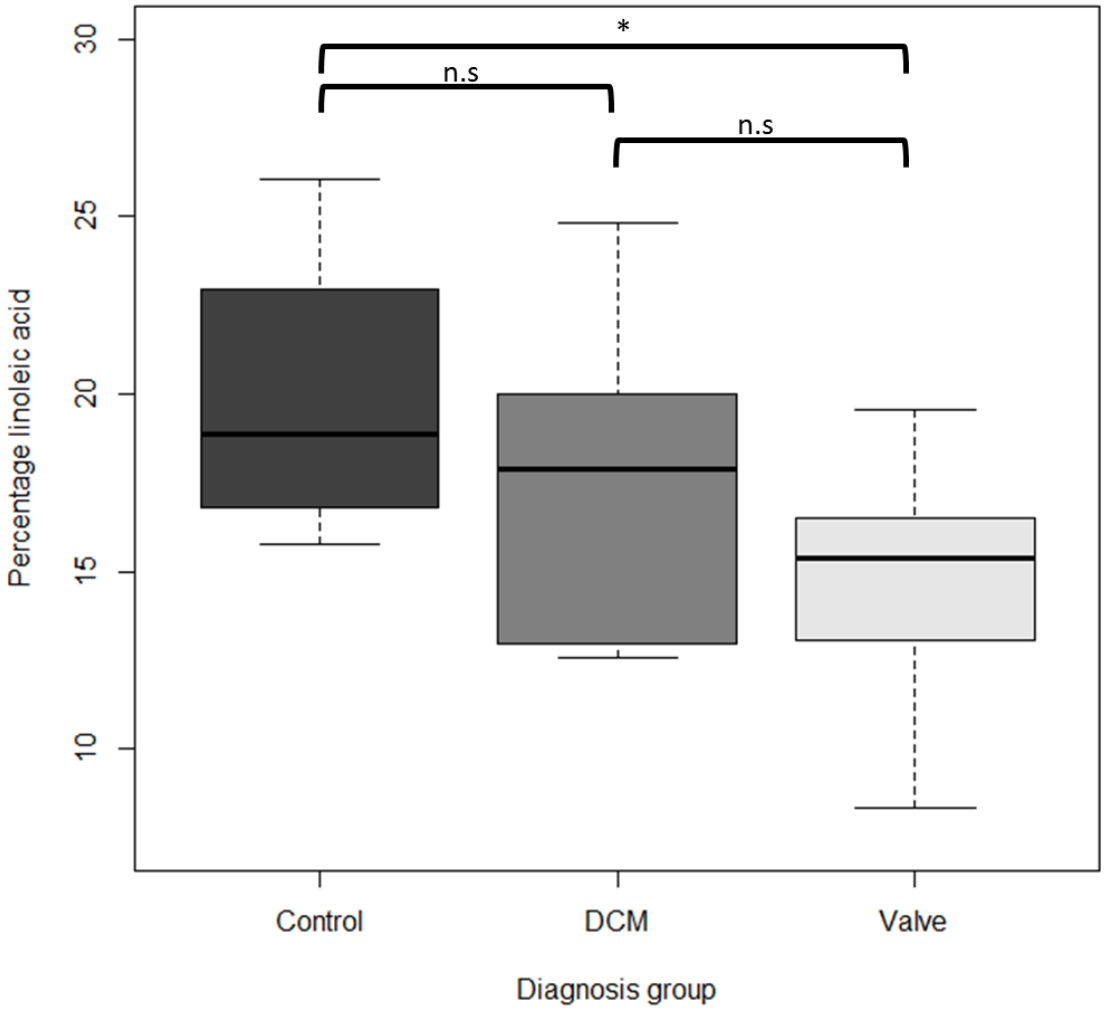


Figure 7.7. Linoleic acid levels (expressed as a percentage of total fatty acid) in each of the three groups - no cardiac diagnosis, DCM and valve disease. Thick centre line = median, upper and lower edges of boxes = upper and lower quartile ranges respectively, whiskers = full range. * = p<0.05, n.s = not significant.

7.4.3.2. Oleic acid and palmitic acid

Palmitic acid is a precursor to oleic acid, see Figure 7.2 (Kanehisa et al., 2016). Both palmitic acid and oleic acid had significant differences in the percentage contribution to fatty acid profiles between groups prior to Bonferroni correction (Table 7.2). The regression in Figure 7.8 shows that there were significant positive relationships between palmitic acid and oleic acid in the DCM group ($F = 5.573$, $p = 0.046$) and valve disease group ($F = 10.24$, $p = 0.007$); and a non-significant positive relationship in the no cardiac diagnosis group ($F = 1.973$, $p = 0.295$). Palmitic acid is also a precursor to arachidic acid (Kanehisa et al., 2016) which also had significant differences between groups prior to Bonferroni

correction. The regression analysis of palmitic acid vs arachidic acid for the DCM and valve disease groups is shown in Figure 7.9. Arachidic acid was only present at a maximum of 0.5% in any individual. In nine individuals arachidic acid was not detected, including all individuals without cardiac diagnoses. The relationship between palmitic and arachidic acids was positive in both the DCM and valve disease groups but not significant (DCM – $F = 1.49$, $p = 0.257$; valve disease – $F = 0.050$, $p = 0.826$).

The significant ANOVA results prior to Bonferroni correction for palmitic acid and oleic acid were due to differences between the valve disease and DCM groups; differences: palmitic acid = 4.22 ($p = 0.0047$), oleic acid = 5.76 ($p = 0.0043$). When the percentages of palmitic acid and oleic acid were combined the ANOVA remained significant ($F = 8.17$, $p = 0.0017$), but the differences between the groups remained only between the valve disease and DCM groups (difference = 9.98, $p = 0.0016$). Figure 7.10 shows the differences between the groups when palmitic and oleic acids were combined.

Only related fatty acids that gave individually significant ANOVA results were considered in pairs. Heptadecanoic acid and docosahexaenoic acid gave significant ANOVA results prior to Bonferroni correction but did not have any related fatty acids which gave significant ANOVA results, therefore no further analyses were performed between those fatty acids.

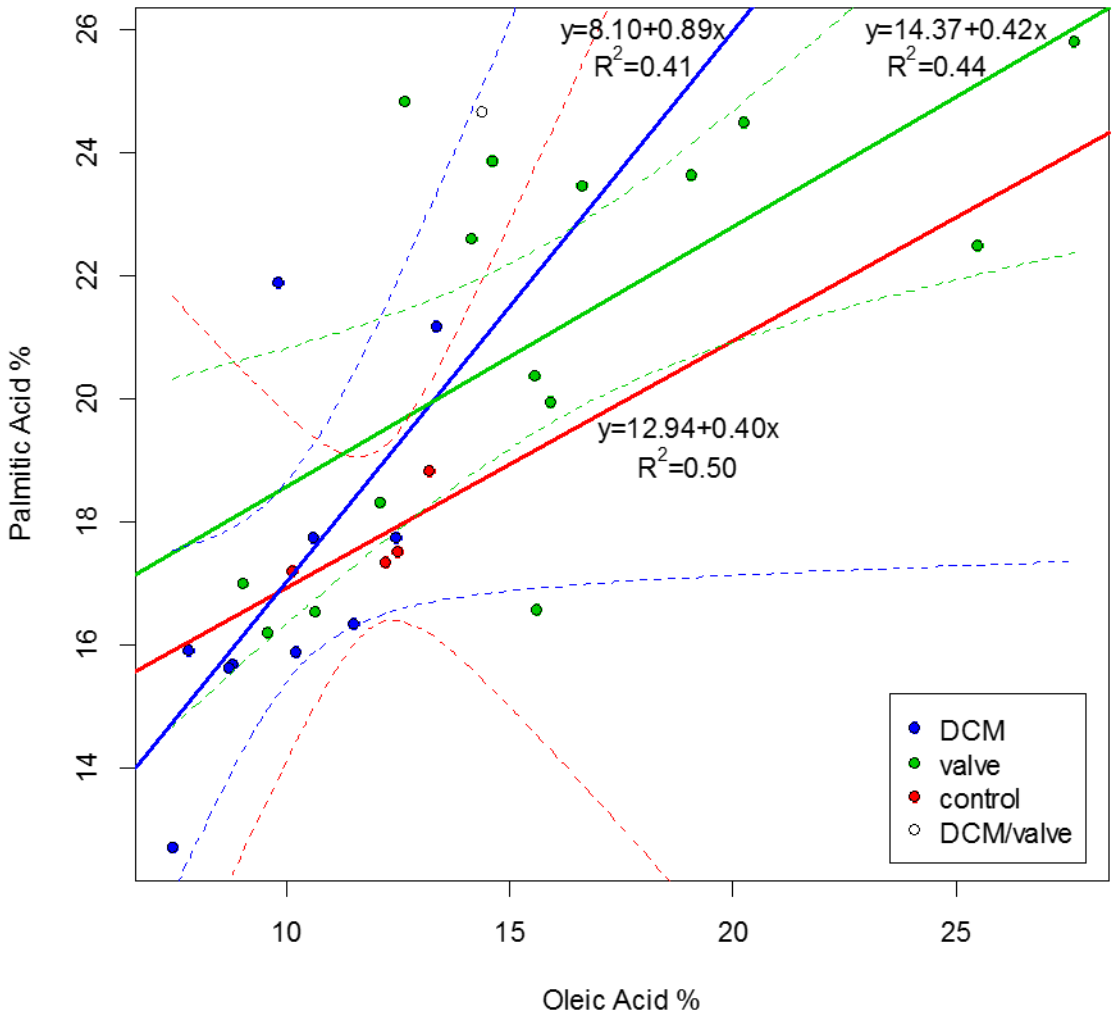


Figure 7.8. Percentage palmitic acid vs percentage oleic acid with associated regression lines. 95% confidence intervals are shown as dashed lines. Individuals with a DCM diagnosis are shown in blue, individuals with a valve disease diagnosis are shown in green, and individuals with other diagnoses are shown in red. The non-coloured point indicates the individual which had its diagnosis recorded as “DCM/end stage valve disease”.

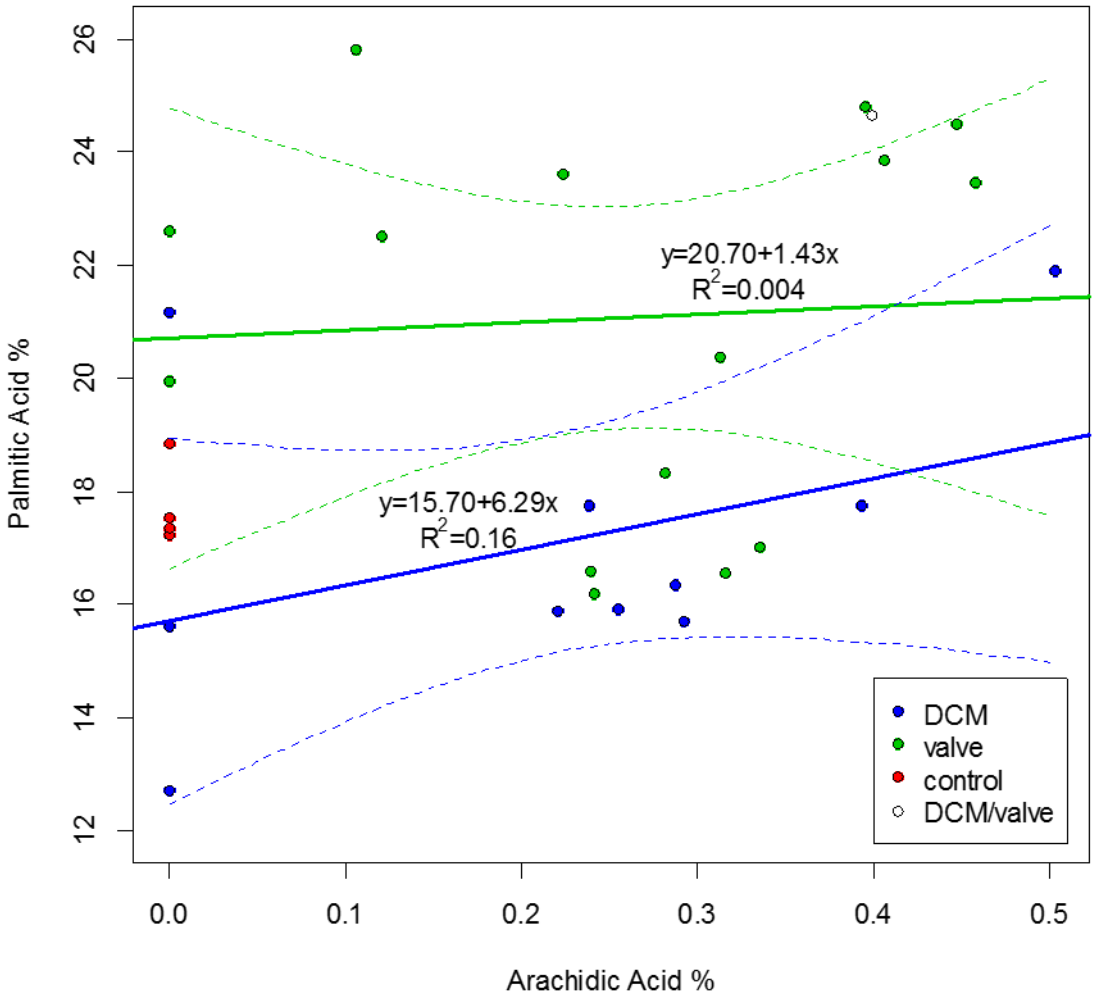


Figure 7.9. Percentage palmitic acid vs percentage arachidic acid with associated regression lines. 95% confidence intervals are shown as dashed lines. Individuals with a DCM diagnosis are shown in blue, individuals with a valve disease diagnosis are shown in green, and individuals with other diagnoses are shown in red. The non-coloured point indicates the individual which had its diagnosis recorded as "DCM/end stage valve disease".

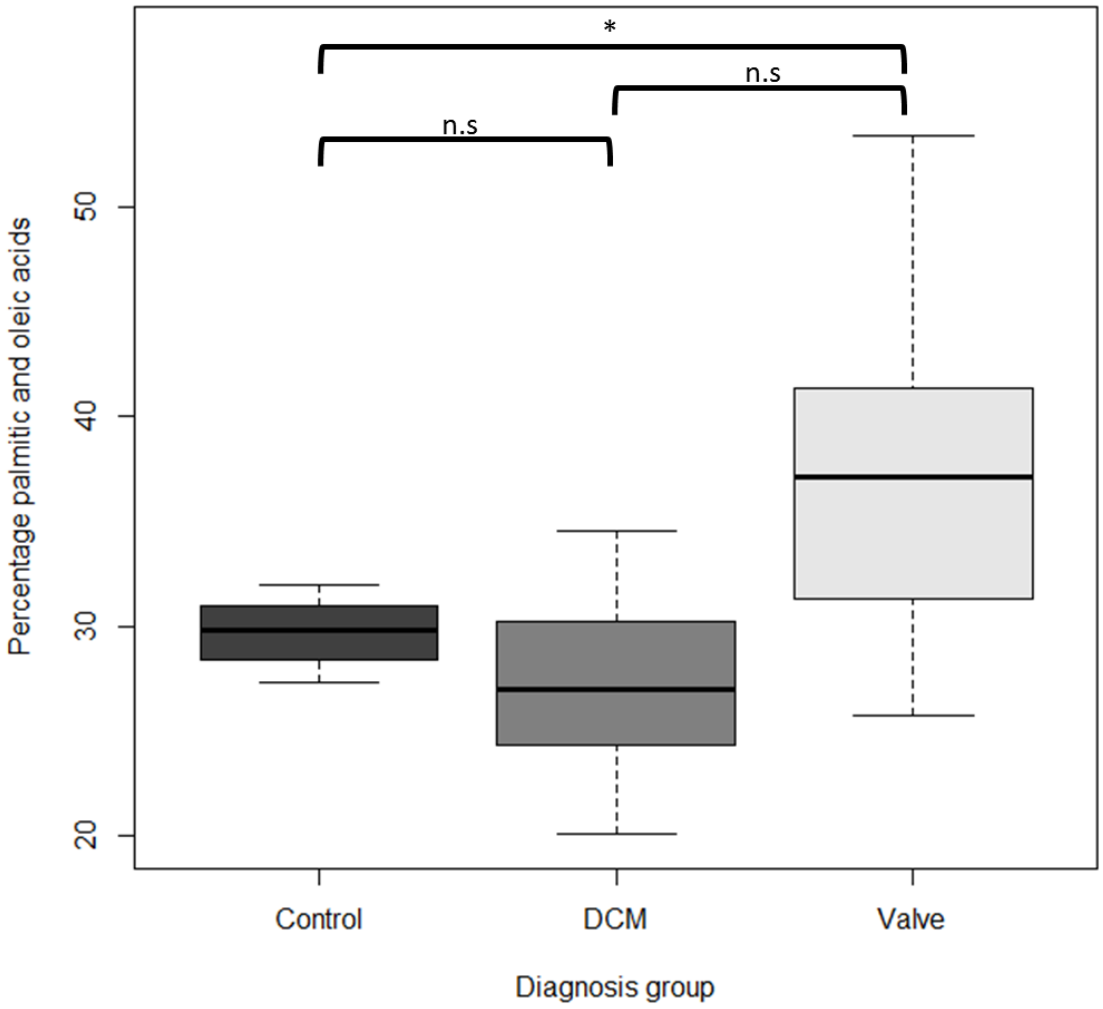


Figure 7.10. Total percentage of fatty acids that were palmitic and oleic acid for each of the three groups - no cardiac diagnosis, DCM and valve disease. Thick centre line = median, upper and lower edges of boxes = upper and lower quartile ranges respectively, whiskers = full range. * = $p<0.05$, n.s = not significant.

The linoleic-arachidonic and palmitic-oleic fatty acid pathways appeared to show opposite effects. The DCM and no cardiac diagnosis groups had a greater contribution to their fatty acid profiles from linoleic acid and arachidonic acid than palmitic and oleic acids, whereas the valve disease group was opposite with a larger contribution from palmitic and oleic acids than linoleic and arachidonic acids (Figure 7.5 and 7.10). Due to this the combined percentages of linoleic acid and arachidonic acid were regressed on the combined palmitic and oleic acid percentage, Figure 7.11. There was a negative relationship between the two fatty acid groups, this was significant within the valve disease group ($F = 36.14$, $p = 0.000044$), but not significant in the DCM ($F = 3.28$, $p =$

0.1077) and no cardiac diagnosis groups (F = 0.03653, p = 0.8661). Combining the percentage of all four fatty acids and testing for a difference between diagnosis groups using ANOVA showed that there was no difference between the groups (F = 1.415, p = 0.26).

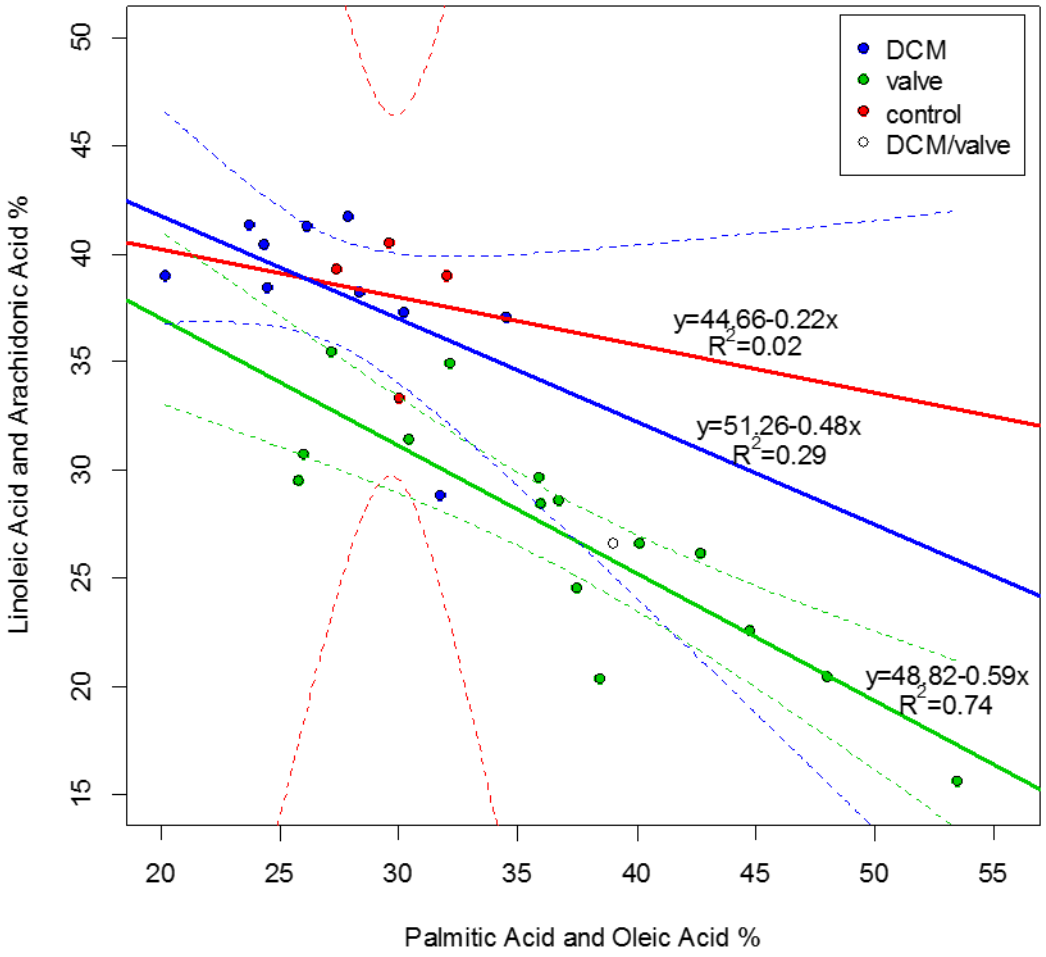


Figure 7.11. Plot of palmitic and oleic acids vs linoleic and arachidonic acids with associated regression lines. 95% confidence intervals are shown as dashed lines. Individuals with a DCM diagnosis are shown in blue, individuals with a valve disease diagnosis are shown in green, and individuals with other diagnoses are shown in red. The non-coloured point indicates the individual which had its diagnosis recorded as “DCM/end stage valve disease”.

7.5. Discussion

Significant differences were identified in the fatty acid profiles in the blood of dogs with valve disease compared to patients with DCM or no cardiac diagnosis. This is surprising as both DCM and valve disease have inflammatory elements in their pathogenesis (Coté et al., 2013, Kühl et al., 1996,

Mavropoulou et al., 2016). In addition all individuals within the no cardiac diagnosis group had diagnoses with inflammatory components (Table 7.1) (Pavletic, 2014, Struglics et al., 2015, Tefferi et al., 2011). Cluster analysis identified two groups based on fatty acid profiles (Figure 7.3); the valve disease individuals predominantly clustered separately from individuals with DCM or no cardiac diagnosis. Interestingly there were two individuals with DCM in the cluster containing the majority of the valve disease patients. Importantly the diagnosis of one of these dogs was not specific and recorded as “DCM/end stage valve disease” and the two diagnoses can be difficult to differentiate (M Cobb personal communication). Based on the fatty acid profile it would seem that this dog had end stage valve disease and not DCM. It is possible the other DCM patient clustering with the valve disease patients may have had undiagnosed valve disease.

There were significant differences in the percentage contributions of several fatty acids to the overall profile between diagnosis groups prior to Bonferroni correction (Table 7.2). Arachidonic acid was the only fatty acid which showed statistically significantly different percentages between diagnosis groups following Bonferroni correction (Table 7.2, $F = 10.2$, $p = 0.0005$). Individuals with valve disease had significantly less arachidonic acid than individuals with DCM (difference = -8.13, $p = 0.0004$), but there was not a significant difference between individuals with DCM and no cardiac diagnoses, or individuals with valve disease and no cardiac diagnosis (Figure 7.6).

Possible reasons for the difference in the percentage of arachidonic acid in the fatty acid profiles between diagnosis groups include (i) differing amounts of arachidonic acid production from its precursors, (ii) differing amounts arachidonic acid release from cell membrane phospholipids and (iii) differing rates of metabolism to downstream products.

If less arachidonic acid was being produced from its precursors it might be expected that there would be increases in the amount of the precursors in the valve disease group compared to the DCM group. The two fatty acids that are immediately upstream from arachidonic acid are gamma linolenic acid

(C18:3n6) and dihomo-gamma-linolenic acid (C20:3n6) (Figure 7.1, (Kanehisa et al., 2016)). Gamma linolenic acid was filtered from the data as only two individuals had a percentage greater than 0.1% in their fatty acid profile. These individuals did not appear different overall to other individuals, they were in separate clusters and no other fatty acids were different in only these individuals. In addition the percentage contribution of gamma linolenic acid to these individuals was low (1.9% and 5.2%). Dihomo-gamma-linolenic acid only contributed a mean of less than 2% to the fatty acid profiles and there were no differences in the percentages between the groups, despite differences between groups in arachidonic acid percentages (Table 7.2). This suggests that the differences in the percentage contribution of arachidonic acid the fatty acid profiles between groups were unlikely to be due to differing amounts being produced.

Arachidonic acid is the precursor to many eicosanoids (Fonteh et al., 1994, Hamberg and Samuelsson, 1974). Eicosanoids have been shown to be involved in pro-inflammatory responses in a range of normal and adverse physiological processes (Harris, 2009, Li et al., 1997b, Raj and Chen, 1987, Vane, 1971). During inflammation arachidonic acid is released from cell membranes by phospholipase A_2 enzymes to allow the production of eicosanoids (Bonventre et al., 1997, Burch et al., 1986, Lee et al., 2010, Xu et al., 2003). All individuals in the present study had diagnoses that involve an inflammatory component (Table 7.1, (Coté et al., 2013, Kühl et al., 1996, Mavropoulou et al., 2016, Pavletic, 2014, Struglics et al., 2015, Tefferi et al., 2011)). If the inflammatory response was the same across all disease processes then a difference in the percentage of arachidonic acid in the blood between diagnosis groups would not be expected. If variations in the release of arachidonic acid from cell membranes is the cause of the differences observed, less arachidonic acid in the blood may suggest that less had been released from tissues. It may be that valve diseases have less of an inflammatory component than DCM and the other non-cardiac diseases (aural haematoma, skin disease, ruptured anterior cruciate, and cancers) with less arachidonic acid available to be converted to eicosanoids. Alternatively phospholipase A_2 enzymes are dependent on free Ca+ concentrations indicating that there may

have been less free Ca+ in individuals with valve disease compared to other diseases (Clark et al., 1991). Indeed the expression of phospholipase A_2 enzymes may vary in different disease processes.

We are not aware of any literature available to either support or refute the third possibility, that differing rates of metabolism of arachidonic acid to downstream products might be occurring. Despite this it is possible that greater amounts of a fatty acid in the blood were a consequence of successful release from cell membranes, but then failure to be converted to downstream products. If fatty acids were released from cells, but not converted to downstream products it is possible that these accumulated in the blood. The second and third explanations for the differing arachidonic acid levels are not mutually exclusive of course, and could have been acting together.

Linoleic acid showed significant differences in the percentage contribution to the fatty acid profiles between groups prior to Bonferroni correction (Table 7.2, $F = 3.54$, $p = 0.043$). Individuals with valve disease had significantly less linoleic acid than individuals with no cardiac disease (Figure 7.7, $p<0.05$). Linoleic acid is obtained exclusively from the diet and it is possible that there were differences between individuals in the amount consumed due to different food fed by owners (Eilander et al., 2015, Johnson et al., 2015). This variation in dietary intake could explain differences between individuals, but while the diets of individuals in the present study were not recorded it seems unlikely that dietary intake would have varied consistently between diagnosis groups, particularly with the range of breeds and ages in each group. One suggestion is that future studies should record the diet that individuals are consuming so that this can be accounted for in order to run a completely controlled trial.

Linoleic acid is a precursor of arachidonic acid (Figure 7.1, (Kanehisa et al., 2016)), therefore it was hypothesised that the differences between diagnosis groups in the percentage of linoleic and arachidonic acids may be due to their relationship. The linear regression revealed that there may be differing metabolism between the two fatty acids between individuals with different diagnoses. There was a positive, albeit not significant, relationship between

arachidonic acid and linoleic acid within individuals with valve disease, and a negative relationship within individuals with DCM (significant, p = 0.023) and individuals with no cardiac diagnosis (not significant) (Figure 7.4). This relationship between the fatty acids led to the additional hypothesis that there might be combined effects of arachidonic acid and linoleic acid, and subsequently of their metabolites, in disease pathogenesis. This was tested by combining the percentages of the two fatty acids and testing for a difference between the groups. The result was of physiological interest as the percentage of the two fatty acids combined was significantly lower in individuals with valve disease compared to individuals with DCM, and individuals with no cardiac diagnosis (p = 0.0000055 and p = 0.00065 respectively), while the difference between individuals with DCM and individuals with no cardiac diagnosis reduced (Figures 7.5 - Figure 7.7). There is evidence in the literature that the metabolites of arachidonic and linoleic acids perform similar biological functions. Metabolites of both fatty acids are reduced in the presence of cyclooxygenase inhibitors indicating conserved metabolic pathways (Kaduce et al., 1989, McAdam et al., 1999). The effects of many of the metabolites are similar with both the arachidonic acid derived eicosanoids and the primary linoleic acid metabolite 9-hydroxyoctadecadienoic acid (9-HODE) exhibiting pro-inflammatory effects (Harris, 2009, Hattori et al., 2008, Vane, 1971). Further indication of the potential synergetic effects of arachidonic and linoleic acid metabolites is that production of 9-HODE is reduced in the presence of arachidonic acid; the production of the metabolites appears to be in direct competition for rate limiting enzymes (Kaduce et al., 1989).

In the fatty acid profiles of valve disease patients in the present study, there was a greater contribution from both palmitic and oleic acids compared to profiles from the DCM and no cardiac diagnosis patients (Table 7.2). Palmitic acid is a precursor of oleic acid; both are on a separate metabolic pathway to linoleic acid and arachidonic acid (Figures 7.1 and 7.2), (Kanehisa et al., 2016). Within and across all groups in the present study, there was a positive relationship between the palmitic and oleic acids (Figure 7.8). If this relationship was due to increased availability of the precursor (palmitic acid), it would be expected that the same relationship would exist between palmitic acid and its

other immediate downstream fatty acid, stearic acid (C18:0), but this was not the case in these patients (Table 7.2). This indicates that the release of palmitic and oleic acids from membrane phospholipids and subsequent conversion to downstream products is related, but independent of the amount of palmitic acid. Combining the percentage of palmitic acid and oleic acid into a single percentage and testing for a difference between the groups, increased the difference between the valve disease and DCM groups (difference = 9.98, p=0.0016), but did not result in a significant difference between the valve disease group and the no cardiac diagnosis group (Table 7.10).

Investigating whether there was a relationship between fatty acid pathway utilisation revealed that there is a concentration dependant relationship between the linoleic acid and palmitic acid pathways (Figure 7.11). This shows that patients with valve disease have a greater contribution to their fatty acid profiles from the palmitic and oleic acid pathway than the linoleic and arachidonic acid pathway compared to individuals with DCM and other diagnoses. This was further supported by combining all four fatty acids (linoleic, arachidonic, palmitic, and oleic acids) and performing an ANOVA which revealed that there were no significant differences in the overall percentages between diagnoses. Previous studies have shown inverse relationships between the linoleic and palmitic acid pathways in mice, chickens and humans implying that the relationship is a general biological phenomenon (Høstmark and Haug, 2014, Lowry and Tinsley, 1966, Rupp et al., 2010). Why one pathway is preferred over the other is not clear, and the results presented in the current study are in contrast to previous results on a similar disease system. Rupp *et al.* (2010) found that human patients with greater left ventricular dilation have a larger percentage of oleic acid and a smaller percentage of arachidonic acid compared to patients with less left ventricular dilation. In the current study the amount of ventricular dilation was not recorded but by definition DCM patients have left ventricular dilation, while valve disease patients can develop dilation but this is not a defining characteristic of the disease (Iung et al., 2003, Oakley, 1978). Based on these definitions it would seem that our results contradict those of Rupp *et al.* (2010) with patients with DCM having a larger percentage of arachidonic acid and a lower percentage of oleic acid than valve

disease patients. This does not affect the validity of dogs as models of disease in people. The most likely explanation for this difference is that Rupp *et al.* (2010) measured fatty acids in serum whereas this current study analysed whole blood. Fatty acids contained within serum are available for conversion to other products or be taken up by cells, and are affected by short term (less than 24 hours) dietary and other environmental changes. In contrast, red blood cells are less affected by short term dietary changes (Harris et al., 2013, Wolters et al., 2014). Importantly in this context, both short and long term fatty acid availability are relevant to disease progression therefore fatty acids in whole blood are likely to be more representative of what was affecting disease progression than fatty acids in either serum or red blood cells individually. An additional possibility for the differences between this current study and Rupp *et al.* (2010) is that none of the patients in the study by Rupp *et al.* (2010) had confirmed diagnoses. Valve disease and coronary artery disease were excluded as the underlying cause of left ventricular dilation, but infarction was not, this may have skewed the fatty acid results due to the strong inflammatory nature of myocardial infarction (Frangogiannis, 2014, Rupp et al., 2010).

Many of the fatty acids analysed contributed only a small percentage (<5%) to the overall fatty acid profiles (Table 7.2), this is consistent with previous reports (Forouhi et al., 2014, Fretts et al., 2014, Smith et al., 2008, Wolters et al., 2014). Three of the fatty acids for which a significant ANOVA result was obtained prior to Bonferroni correction only contributed a small percentage to the fatty acid profiles: heptadecanoic acid, arachidic acid, and docosahexaenoic acid (Table 7.2). Heptadecanoic acid has been associated with a protective effect of diabetes, and women with higher levels are less likely to suffer from myocardial infarction (Forouhi et al., 2014, Warensjö et al., 2010). In the present study, higher levels of heptadecanoic acid were associated with valve disease, while individuals with DCM and non-cardiac diagnoses had lower levels. The mechanistic and biological significance of this finding is unclear. There is some evidence that higher levels of circulating arachidic acid are associated with lower risk of atrial fibrillation and diabetes (Forouhi et al., 2014, Fretts et al., 2014). In the current study all individuals without a cardiac diagnosis lacked arachidic acid in their fatty acid profiles (Figure 7.9). This could be a real effect

or it could be attributable to the relatively low number of individuals in the control “no cardiac diagnosis” group. There also were individuals from the other groups with no aracidic acid in their fatty acid profiles (Figure 7.9). Further work with a larger number of control and affected samples is now warranted to confirm this effect. Docosahexaenoic acid is an ω-3 fatty acid which has been implicated as having beneficial effects in a wide range of diseases including heart disease and neurological dysfunction (D'Ascoli et al., 2016, Eide et al., 2016). In the current study there was less docosahexaenoic acid in the valve disease group compared to the DCM and no cardiac diagnosis group. The result was not significant following the Bonferroni correction and the percentage differences between the groups was small. Despite this docosahexaenoic acid may be of interest in future studies for the anti-inflammatory effects of its metabolites (Bagga et al., 2003, Todoric et al., 2006).

While all other fatty acids measured did not reveal any significant differences between diagnosis groups in the current study, some have previously been associated with types of heart disease. These include increased levels of palmitoleic acid associated with heart failure, higher levels of behenic acid and stearic acid associated with lower risk of developing atrial fibrillation, women with higher circulating pentadecanoic acid are less likely to have a myocardial infarction, hypertensive rats have higher circulating eicosedienoic acid, and in renal patients higher circulating C20:5n3 is associated with good cardiac functional measures (Djoussé et al., 2012, Eide et al., 2016, Fosshaug et al., 2015, Fretts et al., 2014, Kim et al., 2013b, Warensjö et al., 2010, Wu et al., 2011). Despite this there was no evidence for altered levels of any of these fatty acids in the individuals with dilated cardiomyopathy or valve disease studied here.

A previous study examining fatty acids in myocardial tissue of DCM affected and unaffected dogs identified only differential levels of docosatetraenoic acid (Smith et al., 2005). In the current study docosatetraenoic was not measured so it is not possible to directly compare results for this fatty acid. The differences identified between groups in the current study are due to differences between valve disease patients and DCM and non-cardiac patients, not differences

between DCM and non-cardiac patients. Thus the results of the current study are consistent with those of Smith *et al.* (2005).

In addition to the pro-inflammatory activity of specific fatty acids, there is an additional factor potentially involved in disease progression, namely the change in the molecular composition of the cell membranes themselves. Alterations in plasma membranes can affect the biophysical properties of the membranes including permeability and membrane-fluidity which may in turn contribute to the pathologic processes (Burns et al., 1979, Kröger et al., 2014). The primary differences in fatty acid profiles between diseases are due to arachidonic acid which is strongly associated with increased inflammation (Burch et al., 1986, Lee et al., 2010, Xu et al., 2003). This suggests that inflammation is exerting the stronger effect on pathophysiology, but the effect of altering the composition of phospholipid membranes on pathology cannot be discounted.

There are drugs which affect the conversion of fatty acids to downstream products. These include COX inhibitors which inhibit the production of eicosanoids, inhibitors of β-oxidation of fatty acids, and promotors of fatty acid β-oxidation (Buzzai et al., 2005, Kantor et al., 2000, McAdam et al., 1999, Serisier et al., 2006). None of the dogs in the present study were known to have been treated with anti-inflammatory drugs prior to sampling, thus differences in fatty acid profiles were likely to be due to physiological or pathophysiological processes or dietary effects. As previously mentioned, dietary effects were likely to be minimal, therefore the differences observed in fatty acid profiles were most likely to be due to the disease processes themselves. With additional understanding of the contribution to disease of fatty acid profiles, fatty acid metabolism altering drugs could be relevant therapeutic agents in a range of diseases.

7.6. Conclusions

In summary, differences in whole-blood fatty acid profiles were quantified in dogs with cardiovascular disease. In particular the fatty acid profile of dogs with valve diseases was distinct from dogs with other cardiovascular diseases. Additional work is required to establish how the fatty acid profiles of patients

with diseases with inflammatory components compare to healthy individuals. With increased knowledge of how fatty acid profiles relate to disease progression there is potential for different drug therapies to be employed and targeted dietary supplementation of particular fatty acids.

Chapter 8 General discussion and conclusions

Canine diseases are a clinical problem in their own right, but can also be used as models of human diseases (Egenvall et al., 2006, Egenvall et al., 2007, Lenffer et al., 2006, OMIA, 2016, Pelander et al., 2015, Tsai et al., 2007). Many studies utilising dogs as models for human dilated cardiomyopathy have used induced disease processes rather than studying natural disease progression (Gu et al., 2006, Sandusky et al., 1981). Artificially induced disease often only replicates a small aspect of disease and does not reflect the typically longer time scales involved in natural disease progression in both humans and animals (Denayer et al., 2014, van der Worp et al., 2010). Those studies which have made use of spontaneous canine DCM to elucidate genetic causes of the disease have frequently been of poor quality with low numbers of cases and controls, and/or inappropriate controls (Simpson et al., 2015b).

Throughout this thesis more appropriate controls than previous canine genetic studies have been used, although some limitations with regards to controls still existed. In particular examining the fatty acid profiles of canine cardiac patients relied on remnants of blood samples obtained following clinical diagnostic testing. Despite age, breed, sex, and neuter status being matched where possible this was limited and even those patients that were not cardiac patients still had evidence of disease processes. Ideally this fatty acid profile study would have used full age, breed, sex, and neuter status matched affected and unaffected control dogs, but due to the ethical constrains of obtaining blood samples from healthy dogs this was not possible. Blood samples available were remnants taken for veterinary diagnostic purposes, which complies with Home Office regulations and the Veterinary Surgeons Act. There was, however some matching of age, breed, sex, and neuter status between diagnosis groups so the results are still important as they indicate that fatty acid profiles between cardiac diagnoses appear to be different.

For the genetic tests of association (Chapter 4 and Chapter 6) compromises were made as to when to include individuals in the unaffected group. Irish

Wolfhounds are susceptible to developing a number of conditions at a young age and therefore few live unaffected by DCM into old age. Establishing age thresholds for inclusion of individuals in the unaffected groups generated the most robust unaffected by DCM control groups ever used in canine DCM genetic association testing (Chapter 4). By using these robust age restrictions for inclusion in the unaffected control groups the genetic association test results presented in this thesis are the most accurate to date for Irish Wolfhound DCM. Ideally the thresholds would be increased to closer to 95-99% of affected individuals affected before the age restriction, but in Irish Wolfhounds in this thesis this was not practical as there would have been very few individuals in the unaffected control group. The collection and genotyping of additional individuals, both affected and unaffected, would allow for more robust age restrictions to be used.

In this thesis a theoretical model was developed combining the effects of multiple genetic loci on the development of DCM in Doberman Pinchers (Chapter 3) (Simpson et al., 2015a). The most direct test of this model would be to obtain DNA samples from phenotyped Doberman Pinchers and genotype them at the loci in the model. As access to Doberman Pincher DNA samples was limited Irish Wolfhounds were utilised because the breed is also affected by DCM and there have previously been genetic loci associated with the disease (Egenvall et al., 2006, Philipp et al., 2012). The results of this study showed that in Irish Wolfhound DCM combining genotypes at multiple loci leads to stronger genetic associations (Chapter 4, Tables 4.8 – 4.12). These results extend the Doberman Pincher model to other breeds and indicate that genetic testing in other breeds is likely to require multiple loci to be considered together rather than independently. The Doberman Pincher breed would be an ideal starting point to further validate testing multiple loci together. This may also have implications for genetic testing in other diseases and species, including human genetic counselling.

Fatty acid profiling has potential applications as a diagnostic tool, but it requires additional work before it can be utilised in a clinical setting. In this thesis it was established that fatty acid profiles differ between disease processes (0), but due

to the lack of available healthy individuals it is not clear how these fatty acid profiles differ from healthy fatty acid profiles. By establishing normal fatty acid profiles it may be possible to utilise existing drugs such as inhibitors and promotors of fatty acid β-oxidation, and COX inhibitors to modify fatty acid profiles in individuals with disease (Buzzai et al., 2005, Kantor et al., 2000, McAdam et al., 1999, Serisier et al., 2006). Fatty acid profiling may be useful as a diagnostic tool to differentiate between canine DCM and valve disease, which can in some situations be difficult to differentiate (M Cobb personal communication). This may, however, be of limited clinical use as these patients are usually in heart failure and as such treatment is aimed at minimising the effects of the heart failure, and this treatment is typically similar for both diagnoses (Dukes-McEwan, 2000, Häggström et al., 2008).

Fatty acid utilisation as a substrate for ATP generation is a key component of normal cardiac metabolism (Stanley and Chandler, 2002). One of the genetic loci identified as associated with canine DCM is a splice site variant in *PDK4* in Doberman Pinschers (Meurs et al., 2012). This breed was not extensively studied in this thesis, but two Doberman Pinschers with DCM were included in the fatty acid analysis. These two individuals were genotyped at the *PDK4* locus and one was discovered to be heterozygous for the splice site variant while the other was homozygous wildtype. *PDK4* is involved in fatty acid metabolism (Stavinoha et al., 2004) and as such it was hypothesised that the splice site variant may have an effect on the fatty acid profiles. There were some differences in fatty acid profiles between the two Doberman Pinschers, but the greatest effect appeared to be with regards to absolute concentrations of fatty acids. The individual with the *PDK4* splice site variant had the greatest absolute concentrations of fatty acids across all individuals analysed indicating that the *PDK4* variant may be contributing to the DCM phenotype in this individual. This is consistent with Stavinoha *et al.* (2004) who showed that expression of *PDK4* was related to fatty acid utilisation. Further work is required to obtain fatty acid profiles of blood samples from additional genotyped and phenotyped Doberman Pinschers to establish if this potential effect of the *PDK4* variant is as hypothesised. If this hypothesis is validated individuals could be genotyped in advance of developing disease and drugs aimed the *PDK4* variant could be

developed. Even if this mode of action is validated and drugs are developed there remain causes of DCM in Doberman Pinchers to be identified as not all individuals with the disease have the *PDK4* splice site variant.

The length of the polyglutamine tract in the canine *NCOA3* gene had not previously been examined, but based on results presented in this thesis it is not associated with DCM/AF in IWHs or osteosarcoma in IWHs or rottweilers (Chapter 6, Figure 6.1) in the dogs examined and as such is not likely to be the cause of phenotypic variation. The polyglutamine tract in the canine *AR* gene had previously been genotyped in a range of breeds, but not Irish Wolfhounds or Rottweilers, and its length had not been tested for an association with DCM or osteosarcoma in either dogs or people. As with *NCOA3* there was limited variation observed in the *AR* polyglutamine tract (Chapter 6, Figure 6.1), however there was sufficient variation to test for an association with disease. Testing the length of the canine *AR* polyglutamine tract for an association with the development of DCM and osteosarcoma did not reveal any association in either breed tested (Chapter 6). These results do not rule out a role for either androgens or the androgen receptor in the development of canine DCM or canine osteosarcoma, but further work is required to establish their role.

Differential gene expression between affected and unaffected tissue has the potential to identify novel drug targets for a disease, some of which may already have licenced drugs available (Calon et al., 2015, Pfefferle et al., 2016, The Cancer Genome Atlas Research Network, 2014). In this thesis gene expression was measured in canine osteosarcoma tumour tissue and associated non-tumour tissue which identified patterns of gene expression that were differentially expressed in the tumour tissue compared to non-tumour tissue (Chapter 5). A future study for canine DCM would be similar comparisons of affected and unaffected tissue. This would require heart tissue samples from both affected and unaffected dogs. The unaffected dogs in such a study should be sex and breed matched to ensure that any differences identified are due to the disease, not because different breeds are included in the affected and unaffected groups. A study similar to this has been performed by Friedenberg *et al.* (2016), but the unaffected control dogs were not sex and breed matched

so the full potential of such a study was not maximised. Friedenberg *et al.* (2016) identified genes involved in cellular energy metabolism as expressed less in DCM hearts than non-DCM hearts, but this was potentially expected as cellular energy metabolism is known to differ in failing hearts compared to normal hearts (Stanley and Chandler, 2002). Thus the study by Friedenberg *et al.* (2016) does not identify any underlying differences in gene expression that may be the cause as opposed to the result of DCM.

Although the link between canine DCM and canine osteosarcoma is currently only based on the diseases occurring at high prevalence in similar breeds there is additional evidence for potential genetic links between the diseases. These include *SLC2A1*, *MMP3*, and *DKK3*, all genes identified as differentially expressed in osteosarcoma tumours and discussed in Chapter 5. In cardiac disease a key part of disease pathology is that of energy metabolism; fatty acid metabolism is one aspect of this, but the other aspect is glucose (Stanley and Chandler, 2002). Transport of glucose into cells is reliant on cell membrane proteins, of which SLC2A1 is a member (Shows et al., 1987). Within the heart SLC2A1 is responsible for the majority of glucose uptake (Shao and Tian, 2015), thus aberrant expression of the *SLC2A1* gene could contribute to DCM in addition to osteosarcoma. Both MMP3 and DKK3 act on the binding of Wnt activator proteins thus affecting downstream gene transcription (Huelsken and Behrens, 2002, Kessenbrock et al., 2013, Nakamura and Hackam, 2010). *DKK3* expression has been implicated as having a role in DCM disease development, potentially having cardio protective effects during the compensation stage of heart failure (Lu et al., 2016). There is conflicting evidence about the role of DKK3 in osteosarcoma, but expression of DKK3 may ameliorate the effects of the aberrant expression of other genes, potentially with similar effects to those observed in DCM (Lin et al., 2013, Lu et al., 2016, Zhao et al., 2015). It has been shown in patients that have had a myocardial infarction that those with higher *MMP3* expression also had increased morbidity rates (Abd El-Aziz and Mohamed, 2016). Both this thesis and previous studies have shown *MMP3* to be highly expressed in in osteosarcoma tumours (Huang et al., 2016, Tsai et al., 2014). In both DCM and osteosarcoma the effect of MMP3 could be on the degradation of extracellular matrix components or its action on

Wnt transcription factor signalling (Kessenbrock et al., 2013, Okada et al., 1986). MMP3 plasma levels have been shown to be consistently higher in men than women (Collazos et al., 2015). This difference in plasma MMP3 could explain the some of the differences observed between males and females in the development of both osteosarcoma and DCM. These links between canine DCM and osteosarcoma are as yet unproven, but could be promising hypotheses to be tested in future work.

The canine models of osteosarcoma and DCM/AF have the potential to yield further results to increase understanding of both diseases in dogs and people. One limitation of the current study is that additional affected and older unaffected individuals are required to improve the robustness of any conclusions drawn. The current longitudinal health study is continuing and many individuals enrolled in the study may ultimately be included in either the affected or older unaffected groups for association testing. As with any longitudinal health study the value of the data will increase over time. There remain very few genetic loci associated with canine DCM and osteosarcoma. Future work could include whole genome or exome sequencing of affected individuals, using older unaffected individuals as controls. Any additional loci identified could be incorporated into the multigenic models reported in this thesis to inform preventative medicine and breeding practices in the canine population. In addition novel loci identified as associated with disease in dogs have the potential to inform human genetic counselling.

In conclusion this thesis has shown that no single genetic factor influences canine DCM or osteosarcoma. With multiple loci implicated in the development of DCM in Irish Wolfhounds, the methodology developed could be utilised in a range of species and diseases. The identification of somatic genetic causes of disease could lead to improved welfare through selective breeding in animals, improved genetic counselling in people, novel treatments identified in both humans and animals, and potential gene therapy in all species. Where somatic genetic causes of disease have not been identified differential gene expression analyses could identify potential novel therapeutic targets to treat diseases.

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Appendix 1

Genetics of Human and Canine Dilated Cardiomyopathy

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Review Article

Genetics of Human and Canine Dilated Cardiomyopathy

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Cardiovascular disease is a leading cause of death in both humans and dogs. Dilated cardiomyopathy (DCM) accounts for a large number of these cases, reported to be the third most common form of cardiac disease in humans and the second most common in dogs. In human studies of DCM there are more than 50 genetic loci associated with the disease. Despite canine DCM having similar disease progression to human DCM studies into the genetic basis of canine DCM lag far behind those of human DCM. In this review the aetiology, epidemiology, and clinical characteristics of canine DCM are examined, along with highlighting possible different subtypes of canine DCM and their potential relevance to human DCM. Finally the current position of genetic research into canine and human DCM, including the genetic loci, is identified and the reasons many studies may have failed to find a genetic association with canine DCM are reviewed.

1. Dilated Cardiomyopathy Aetiology and Epidemiology

Cardiovascular disease is the fourth most common cause of death in dogs [1] and one of the most common causes of death in humans [2]. Dilated cardiomyopathy (DCM) is the second most prevalent form of heart disease in dogs, accounting for 10% of cardiac diagnoses [3], and is estimated to be the third most common inherited type of heart disease in humans, reported to affect 35.6 in 100,000 people, although this is thought to be an underestimation [4, 5].

Due to the similar nature of DCM in humans and dogs in terms of disease phenotype and progression, it has been suggested that canine DCM can act as a model for human DCM [6]. Conversely, knowledge obtained from the clinical management of people with DCM may guide improvements in the clinical care and outcomes of companion animals with DCM.

Animal models of DCM are useful in providing insights into the molecular and cellular progression of the disease and thus lead to potential new treatments [7]. While there are many animal models where DCM is induced, for example [8–11], naturally occurring cases of canine DCM are also valuable, in particular with regard to natural disease progression, especially where the underlying cause can be shown to be similar in dogs and people (e.g., similar genetic function) [12]. In addition to providing a potential natural model for human DCM, canine cardiovascular health is an important issue in its own right. Understanding the disorder will impact veterinary care, treatment, and prognosis and may also influence pedigree breeding, health, and welfare. Here we review the clinically distinct types of canine DCM and relate these to clinical heterogeneity seen in human DCM. Furthermore we provide a review of the known genetic contributions to DCM and discuss how these factors may inform future clinical management and breeding strategies in the dog.

2. Clinical Characteristics of DCM

DCM is characterised by cardiac ventricular chamber enlargement and systolic dysfunction which often leads

Author contribution

Siobhan Simpson performed the literature review, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Jennifer Edwards and Thomas F. N. Ferguson-Mignan contributed towards the literature review, reviewed drafts of the paper.

Malcolm Cobb, Nigel P. Mongan, and Catrin S. Rutland provided project supervision, reviewed drafts of the paper, funding.

Abstract

Cardiovascular disease is a leading cause of death in both humans and dogs. Dilated cardiomyopathy (DCM) accounts for a large number of these cases, reported to be the third most common form of cardiac disease in humans and the second most common in dogs. In human studies of DCM there are more than 50 genetic loci associated with the disease. Despite canine DCM having similar disease progression to human DCM studies into the genetic basis of canine DCM lag far behind those of human DCM. In this review the aetiology, epidemiology, and clinical characteristics of canine DCM are examined, along with highlighting possible different subtypes of canine DCM and their potential relevance to human DCM. Finally the current position of genetic research into canine and human DCM, including the genetic loci, is identified and the reasons many studies may have failed to find a genetic association with canine DCM are reviewed.

1. Dilated Cardiomyopathy Aetiology and Epidemiology

Cardiovascular disease is the fourth most common cause of death in dogs (Fleming et al., 2011) and one of the most common causes of death in humans (Mathers and Loncar, 2006). Dilated cardiomyopathy (DCM) is the second most prevalent form of heart disease in dogs, accounting for 10% of cardiac diagnoses (Egenvall et al., 2006), and is estimated to be the third most common inherited type of heart disease in humans, reported to affect 35.6 in 100,000 people, although this is thought to be an underestimation (Hershberger et al., 2010a, Raju et al., 2011).

Due to the similar nature of DCM in humans and dogs in terms of disease phenotype and progression, it has been suggested that canine DCM can act as a model for human DCM (Mausberg et al., 2011). Conversely, knowledge obtained from the clinical management of people with DCM may guide improvements in the clinical care and outcomes of companion animals with DCM.

Animal models of DCM are useful in providing insights into the molecular and cellular progression of the disease and thus lead to potential new treatments (Dixon and Spinale, 2009). While there are many animal models where DCM is induced, for example (Akar et al., 2005, Argenziano and Dickstein, 2000, Huntington et al., 1998, Toyoda et al., 1998), naturally occurring cases of canine DCM are also valuable, in particular with regard to natural disease progression, especially where the underlying cause can be shown to be similar in dogs and people (e.g., similar genetic function) (Hongo et al., 1997). In addition to providing a potential natural model for human DCM, canine cardiovascular health is an important issue in its own right. Understanding the disorder will impact veterinary care, treatment, and prognosis and may also influence pedigree breeding, health, and welfare. Here we review the clinically distinct types of canine DCM and relate these to clinical heterogeneity seen in human DCM. Furthermore we provide a review of the known genetic contributions to DCM and discuss how these factors may inform future clinical management and breeding strategies in the dog.

2. Clinical Characteristics of DCM

DCM is characterised by cardiac ventricular chamber enlargement and systolic dysfunction which often leads to congestive heart failure and death (Guttmann et al., 2014). The aetiology of DCM is complex in that genetic factors, myocardial ischemia, hypertension, toxins, infections, and metabolic defects have been implicated in human disease (McNally et al., 2013). Both human and canine DCM have a number of phases of progression starting with a long asymptomatic period before clinical signs appear (Mahon, 2005, Mausberg et al., 2011). During this asymptomatic period, no functional changes in cardiac tissue have yet been reported, but it is possible that the underlying causes (e.g., genetic factors, toxins, and infections) are already initiating the disease (Mausberg et al., 2011). During the next stage, there are again no reported outward clinical signs and the individual usually appears to be healthy, but cardiovascular electrical and morphological changes can be observed (Baig et al., 1998, Brownlie and Cobb, 1999, Mahon, 2005, Mausberg et al., 2011). Cardiovascular electrical changes may be detected using Holter monitoring for

24 hours, with individuals that go on to develop canine DCM often displaying ventricular arrhythmias (Dukes-McEwan et al., 2003). Echocardiography can identify individuals that have an enlarged left ventricle which ultimately leads to symptomatic canine DCM (Dukes-McEwan et al., 2003). Due to the apparently asymptomatic nature of this stage it is often termed the occult or preclinical stage and can last for several years in dogs (Brownlie and Cobb, 1999, Dukes-McEwan et al., 2003, Mausberg et al., 2011). In the final stage of DCM patients present with clinical signs of heart failure, commonly including cough, depression, dyspnoea, weight loss, and syncope, the individual requires treatment for heart failure, but prognosis is often poor (Mausberg et al., 2011, Tidholm et al., 2001). In humans, mortality 10 years following diagnosis is roughly 40%, although there is a wide variation with some individuals remaining asymptomatic, conversely many individuals suffer from sudden death (Lehrke et al., 2011). Dogs also have significantly shortened lifespan following diagnosis, mean survival time following diagnosis, usually at the point of developing overt clinical symptoms, being 34 weeks, although, similar to humans, large variations are observed, with some surviving for several months while others only live for a few weeks (Martin et al., 2010, Petric et al., 2002, Vollmar, 2000).

Treatment of DCM in humans is aimed at minimising the effect of heart failure on the patient and delaying disease progression (Jefferies and Towbin, 2010). Standard medical treatment for human DCM consists of ACE inhibitors and β-blockade, often with a diuretic agent and, in the latter stages of disease progression, inotropic agents are frequently prescribed (Jefferies and Towbin, 2010, Merlo et al., 2014). Heart transplants are often the last resort in treating human heart disease; however the proportion of heart transplants in humans due to nonischemic cardiomyopathy, of which DCM is the second most common form, has increased to become the leading cause of heart transplant in recent years: 51% of transplant cases had nonischemic cardiomyopathy (Taylor et al., 2009). Canine DCM is treated in a similar manner to human DCM, in that treatment is aimed at minimising the effect of heart failure (Dukes-McEwan, 2000). This treatment usually consists of diuretics, ACE inhibitors,

positive inotropes, and other vasodilators (Borgarelli et al., 2001, Dukes-McEwan, 2000). There is evidence that treatment when preclinical symptoms appear can increase lifespan, but this requires screening of individuals for preclinical DCM (Summerfield et al., 2012). Heart transplants and other cardiac assist devices are not generally available to canine DCM patients.

2.1. Evidence for Different Types of Canine DCM

Although dogs within all breeds have the potential to develop DCM, there are some breeds that are particularly afflicted by DCM (Tidholm, 1997). These include Newfoundlands, St. Bernards, Doberman Pinschers, Great Danes, Irish Wolfhounds, Boxers, and English Cocker Spaniels (Egenvall et al., 2006). While these breeds, as well as other less frequently affected breeds, can be diagnosed as having DCM, there is evidence that different breeds may present with distinct types of DCM. This evidence consists of differential survival times from diagnosis, histopathology, inheritance patterns, and age of onset (Calvert et al., 1997, Meurs et al., 2001b, Meurs et al., 2007, Petric et al., 2002, Sleeper et al., 2002, Tidholm and Jonsson, 2005).

Within canine DCM, two distinct types of histopathological variations have been described: “attenuated wavy fibre type” and “fatty infiltration type” (Tidholm and Jonsson, 2005). While this evidence may be subjective, it adds to the evidence suggesting that there are different types of canine DCM. The fatty infiltration type is less subjective and has only been reported in Doberman Pinschers, Estrela Mountain Dogs, Great Danes, and Boxers (Aupperle et al., 2014, Lobo et al., 2010, Stephenson et al., 2012, Tidholm and Jonsson, 2005); while the wavy fibre type is more ubiquitous, it does not seem to be restricted to specific breeds and can also occur in breeds which display the fatty infiltration type (Aupperle et al., 2014, Tidholm and Jonsson, 2005). As the wavy fibre type is found across breeds and in many individuals, it could be the tissue’s response to the other processes of DCM. In particular atrophy, or attenuation, of muscle fibres is a frequent result of processes that prevent normal contractile ability: contractile ability is consistently compromised in DCM (Tidholm et al., 2001). The prevalence and clinical significance of these histopathological variants

remain to be established, although the phenotype can only be established post mortem and thus is unlikely to be useful in a clinical setting.

Human DCM is generally inherited in an autosomal dominant fashion (Posafalvi et al., 2012), but autosomal recessive, X-linked recessive, and mitochondrial modes of inheritance have all been reported (Dec and Fuster, 1994). In common with human inheritance patterns there are several types of inheritance suggested in canine DCM. These include autosomal recessive (Sleeper et al., 2002), X-linked (Meurs et al., 2001b), and the common autosomal dominant inheritance (Meurs et al., 2007, Stephenson et al., 2012); although often with reduced penetrance, not all dogs with the DCM genotype will develop the disease (Distl et al., 2007, Dukes-McEwan and Jackson, 2002). X-linked and autosomal inheritance patterns show that the genetic basis of the disease is different. Recessive and dominant inheritance patterns also suggest the presence of different mutations leading to DCM and reduced penetrance indicates that there are likely to be additional factors involved in the formation of the disease phenotype. These additional factors may involve additional genes, epigenetic effects, and environmental effects including, but not limited to, diet, exercise, stress and toxins, or a combination of any number of these.

There is a wide variation in the long term prognosis of canine DCM. Some dogs, with appropriate disease management, can have a good quality of life for many years following a DCM diagnosis, whereas others die within weeks despite medical intervention (Martin et al., 2010, Petric et al., 2002, Vollmar, 2000). Within this variation there are prognosis trends within breeds. Doberman Pinschers are a breed with particularly poor prognosis, and mean time to death (from diagnosis) is in the range of 7.4 to 9.7 weeks (Calvert et al., 1997, Petric et al., 2002) while the mean time for other breeds is reported to be about four times that at 34 weeks (Petric et al., 2002). Great Danes also suffer from a poor prognosis with Martin *et al.* (2010) finding that they have the lowest median survival time of breeds included in their analysis, while Doberman Pinschers had the lowest upper quartile range.

Age of onset can also affect prognosis. There is a juvenile form of DCM in Portuguese water dogs, where age of onset is measured in weeks from birth (Dambach et al., 1999, Werner et al., 2008), while in most other cases age of onset is measured in years (Martin et al., 2009). It would seem from this that DCM in Portuguese water dogs is a distinct condition. Even within adult canine DCM there is variation between breeds as to when individuals present with outward clinical signs. For example, Great Dane mean age of onset is 4.8 (SD ± 2.3) years (Meurs et al., 2001b), which is comparable to Irish Wolfhound mean age of onset of 4.40 (SD ± 2.03) years (Distl et al., 2007); however, Doberman Pinscher's mean age of onset is in 7.3 years in males and 8.6 years in females (Calvert et al., 1997). This variation in mean age of onset could further suggest that there are different types of canine DCM.

There also appears to be different types of human DCM, with different inheritance patterns and age of onset reported (Mestroni et al., 1999). If canine DCM can be appropriately matched to human DCM in terms of age of onset, inheritance pattern, survival time, and histopathology, they could provide appropriate models for each other. In particular some cases of childhood DCM have been shown to have an autosomal recessive pattern of inheritance (Towbin et al., 2006), and in this instance the juvenile DCM observed in Portuguese water dogs (Dambach et al., 1999) could be an appropriate model. There are currently several types of DCM identified in humans (Posafalvi et al., 2012), but additional studies of canine DCM phenotypes are required to allow appropriate matching of canine and human DCM categories. Once identified, knowledge about canine DCM types could benefit current and future potential treatments and support for both human and canine DCM patients, in addition to elucidating other clinically important factors in canine DCM, such as longevity and prognosis.

2.2. Genomic Research of DCM in Humans

While there are many implicated causes or risk factors related to developing DCM and disease progression, genetics is a common one, with the disease often affecting several individuals within a family. To date mutations in over 50 genes have been associated with DCM in humans; however mutations in the

most prevalent DCM related genes only account for approximately 50% of patients with DCM (Posafalvi et al., 2012). Genetic testing of individuals related to DCM patients can allow those that are at high risk of developing DCM to be more closely monitored (Jacoby and McKenna, 2012). This genetic testing is carried out on a panel of about 50 loci and more than one locus can be implicated in the disease (McNally et al., 2013) suggesting a dose effect, whereby the more DCM alleles an individual carries, the more severe the phenotype (Posafalvi et al., 2012). Gene penetrance has also been reported to affect disease expression and severity, and likewise the type of mutation and the specific gene which is affected often lead to differing features, age of onset or severity, and prognosis (Mestroni and Taylor, 2013, Morales and Hershberger, 2013).

Human DCM-associated genes identified to date are involved in a range of functions but can usually be placed into one of six functional groups: sarcomeric protein genes, cytoskeletal protein genes, nuclear envelope protein, desmosomal protein genes, calcium/sodium-handling genes, and transcription factor genes (Posafalvi et al., 2012). Cardiac muscle consists of striated muscle, and the sarcomere is the smallest unit of contractile muscle within this and thus alterations to this could lead to heart disease (Hamdani et al., 2008). The cytoskeleton forms the majority of the cytoplasm, enabling cells to maintain their shape and facilitating communication within the cell (Frixione, 2000, Hein, 2000). The nuclear envelope provides a barrier between nucleic acid synthesis and the rest of the cell but must remain permeable to allow the cell to function (Macara, 2001), a large number of proteins within the nuclear envelope have been implicated in chromatin organization and gene regulation (Hetzer, 2010). The desmosome provides mechanical strength to tissues and potentially has cell signalling capacity, both of which are essential for cardiac function (Green and Gaudry, 2000). Na^{+}/Ca^{2+} are important in the contraction of muscle (DiPolo and Beaugé, 2006) and as such calcium/sodium-handling genes are important in maintaining the correct concentration of Na^{+}/Ca^{2+} for contraction of the heart. Transcription factors regulate the rate at which transcription of DNA to mRNA occurs; this rate is important in controlling the expression of genes and

therefore the amount of a protein produced (Latchman, 1997). The breakdown of any of these functions has the capacity to lead to disease, including DCM. Table 1 shows the genes with mutations associated with DCM in humans, including the group into which the gene falls (where appropriate).

Table 1. Genes with mutations associated with DCM in humans.

Gene	Location/role	Reference
ABCC9	Calcium/sodium-handling	(Bienengraeber et al., 2004)
ACTC1	Sarcomere & cytoskeleton	(Olson, 1998)
ACTN2	Sarcomere & cytoskeleton	(Mohapatra et al., 2003)
ANKRD1	Sarcomere & transcription factor	(Moulik et al., 2009)
BAG3	Sarcomere	(Arimura et al., 2011, Norton et al., 2011, Villard et al., 2011)
CAV3	Other	(Catteruccia et al., 2009)
CHRM2	Other	(Zhang et al., 2008)
CRYAB	Cytoskeleton	(Inagaki et al., 2006)
CSRP3	Sarcomere & cytoskeleton	(Mohapatra et al., 2003)
CTF1	Other	(Erdmann et al., 2000)
DES	Cytoskeleton	(Li et al., 1999, Schaper et al., 1991)
DMD	Cytoskeleton	(Muntoni et al., 1993, Ortiz-Lopez et al., 1997)
DNAJC19	Other	(Davey et al., 2006)
DOLK	Other	(Lefeber et al., 2011)
DSC2	Desmosome	(Elliott et al., 2010)
DSG2	Desmosome	(Posch et al., 2008)
DSP	Desmosome	(Norgett, 2000)
EYA4	Other	(Schönberger et al., 2005)
FHL2	Sarcomere & cytoskeleton	(Arimura et al., 2007)
FKTN	Cytoskeleton	(Murakami et al., 2006)
FKRP	Cytoskeleton	(Müller et al., 2005)
FOXD4	Transcription factor	(Minoretti et al., 2007)
GATAD1	Other	(Theis et al., 2011)
HCG22	Other	(Meder et al., 2014)
HLA-DQB1	Other	(Pankuweit et al., 2013)
HSPB7	Other	(Stark et al., 2010)
ILK	Cytoskeleton	(Knöll et al., 2007)
LAMA2	Other	(Carboni et al., 2011)
LAMA4	Cytoskeleton	(Knöll et al., 2007)
LAMP2	Other	(Maron et al., 2009)
LDB3	Sarcomere & cytoskeleton	(Arimura et al., 2004)
LMNA	Nuclear envelope	(Fatkin et al., 1999)
MURC	Other	(Rodriguez et al., 2011)
MYBPC3	Sarcomere	(Daehmlow et al., 2002, Hershberger et al., 2010b)
MYH6	Sarcomere	(Carniel et al., 2005, Hershberger et al., 2010b)
MYH7	Sarcomere	(Daehmlow et al., 2002)
MYPN	Cytoskeleton	(Duboscq-Bidot et al., 2008)
NEBL	Sarcomere	(Purevjav et al., 2010)
NEXN	Sarcomere	(Hassel et al., 2009)
NOS3	Other	(Matsa et al., 2013)
PKP2	Desmosome	(Elliott et al., 2010)
PLN	Calcium/sodium-handling	(Haghighi et al., 2003)
PRDM16	Transcription factor	(Arndt et al., 2013)
PSEN1	Other	(Li et al., 2006)
PSEN2	Other	(Li et al., 2006)

RBM20	Other	(Brauch et al., 2009)
RYR2	Calcium/sodium-handling	(Bhuiyan et al., 2007)
SCN5A	Calcium/sodium-handling	(McNair et al., 2004)
SDHA	Other	(Levitas et al., 2010)
SGCD	Cytoskeleton	(Tsubata et al., 2000)
SYNE1	Nuclear envelope	(Zhang et al., 2007)
TAZ	Other	(Bione et al., 1996)
TBX20	Transcription factor	(Kirk et al., 2007)
TCAP	Sarcomere & cytoskeleton	(Hayashi et al., 2004)
TMPO	Nuclear envelope	(Taylor et al., 2005)
TNNC1	Sarcomere	(Hershberger et al., 2010b)
TNNI3	Sarcomere	(Murphy et al., 2004)
TNNT2	Sarcomere	(Kamisago et al., 2000)
TPM1	Sarcomere	(Hershberger et al., 2010b)
TXNRD2	Other	(Sibbing et al., 2011)
TTN	Sarcomere & cytoskeleton	(Gerull et al., 2002)
VCL	Sarcomere & cytoskeleton	(Olson, 2002)
ZBTB17	Other	(Li et al., 2013)

2.3. Genetics of Canine DCM

Canine DCM has often been used as a model for human DCM, but it is also a major clinical challenge in companion animals (Dukes-McEwan et al., 2003, Egenvall et al., 2006, Vollmar, 2000, Wess et al., 2010). It is has been established that, in common with human DCM, canine DCM frequently has a familial basis (Distl et al., 2007, Meurs et al., 2001b, Meurs et al., 2007, Sleeper et al., 2002). Despite this, current understanding of the genetics of canine DCM is limited, in particular compared to the depth of genetic information available for human DCM. Indeed it is only recently that any loci have been associated with canine DCM (Mausberg et al., 2011, Meurs et al., 2012, Meurs et al., 2013, Philipp et al., 2012). Genes associated with canine DCM are DMD in German short-haired pointers (Schatzberg et al., 1999), PDK4 in Doberman Pinschers (Meurs et al., 2012), and STRN in Boxers (Meurs et al., 2013), in addition to a locus on chromosome 5 in Doberman Pinschers (Mausberg et al., 2011). Additional polymorphisms on chromosomes 1, 10, 15, 17, 21, and 37 have also been implicated in Irish Wolfhounds (Philipp et al., 2012). There are two methods that have been employed in attempts to identify genes associated with canine DCM, candidate gene studies, and genome wide association studies (GWAS).

3. Canine Candidate Gene Studies

Candidate gene studies for canine DCM primarily involve examining genes with variants associated with human DCM or associated conditions, for example (Meurs et al., 2008, Philipp et al., 2007, Stabej et al., 2004, Stabej et al., 2005b, Wiersma et al., 2007, Wiersma et al., 2008). The majority of canine DCM genetic studies have been of this type; however, only one mutation associated with canine DCM has been identified in this manner, which is that of a deletion in the Striatin gene in Boxers, a gene previously associated with Boxer arrhythmogenic right ventricular cardiomyopathy using GWAS (Meurs et al., 2013). All other candidate gene studies have failed to find an association with canine DCM in the cohort examined (see Table 2), and unfortunately the small sample sizes frequently utilised could have limited the power to detect an association. In addition to small sample sizes in a number of studies, control (non-DCM cases) dogs have been limited or have not been appropriate (see Table 2 for exact numbers). Suitable controls should be breed matched and over a certain age to ensure that they are unlikely to develop DCM. Table 2 shows the genes examined for mutations associated with canine DCM in a variety of breeds, sample sizes, and control dogs, in the published literature to date.

Table 2. All genes investigated in relation to canine DCM.

Gene	Associated with DCM in humans	Associated with canine DCM	DCM dogs		Control dogs	Human reference	Canine study reference
			Number	Breed			
ACTC1	Y	N	16	Doberman Pinscher	12 mixed breeds	Andreasen *et al.* (2013), Posafalvi *et al.* (2012)	Meurs *et al.* (2001a)
			64	Irish Wolfhound	25 Irish Wolfhounds		Philipp *et al.* (2008a)
			38	Newfoundland	36 Newfoundlands		Wiersma *et al.* (2008)
			5	Doberman Pinscher	5 unspecified dogs without overt heart disease		O'Sullivan *et al.* (2011)
ACTN2	Y	N	5	Doberman Pinscher	5 unspecified dogs without overt heart disease	Andreasen *et al.* (2013), Posafalvi *et al.* (2012)	O'Sullivan *et al.* (2011)
CAV1	N	N	38	Newfoundland	36 Newfoundlands	na	Wiersma *et al.* (2008)
CSRP3	Y	N	64	Irish Wolfhound	25 Irish Wolfhounds	Andreasen *et al*. (2013), Posafalvi *et al*. (2012)	Philipp *et al.* (2008a)
			38	Newfoundland	36 Newfoundlands		Wiersma *et al.* (2008)
			5	Doberman Pinscher	2 Labradors		Meurs *et al.* (2008)
			5	Doberman Pinscher	5 unspecified dogs without overt heart disease		O'Sullivan *et al.* (2011)
DES	Y	N	25	Doberman Pinscher	10 Doberman Pinschers	Andreasen *et al.* (2013), Posafalvi *et al*. (2012)	Meurs *et al.* (2007)
			64	Irish Wolfhound	25 Irish Wolfhounds		Philipp *et al.* (2008a)
			18	Doberman Pinscher	10 Doberman Pinschers		Stabej *et al.* (2004)
			38	Newfoundland	36 Newfoundlands		Wiersma *et al.* (2008)
DMD	Y	Y	2	German short-haired pointers	2 German short-haired pointers with reduced dystrophin	Andreasen *et al.* (2013), Posafalvi *et al.* (2012)	Schatzberg *et al.* (1999)
LDB3	Y	N	38	Newfoundland	36 Newfoundlands	Andreasen *et al*. (2013), Posafalvi *et al.* (2012)	Wiersma *et al.* (2008)
LMNA	Y	N	38	Newfoundland	36 Newfoundlands	Andreasen *et al*. (2013), Posafalvi *et al*. (2012)	Wiersma *et al.* (2008)
			5	Doberman Pinscher	2 Labradors		Meurs *et al.* (2008)
MYBPC3	Y	N	5	Doberman Pinscher	5 unspecified dogs without overt heart disease	Posafalvi *et al.* (2012), Andreasen *et al.* (2013)	O'Sullivan *et al.* (2011)
MYH7	Y	N	38	Newfoundland	36 Newfoundlands	Posafalvi *et al.* (2012), Andreasen *et al.* (2013)	Wiersma *et al.* (2008)
			5	Doberman Pinscher	2 Labradors		Meurs *et al.* (2008)
			5	Doberman Pinscher	5 unspecified dogs without overt heart disease		O'Sullivan *et al.* (2011)
PDK4	N	Y	66	Doberman Pinscher	66 Doberman Pinschers +100 others from 11 breeds	na	Meurs *et al.* (2012)

PLN	Y	N	25	Doberman Pinscher	10 Doberman Pinschers	Posafalvi *et al.* (2012), Andreasen *et al.* (2013)	Meurs *et al.* (2007)
			2 Doberman Pinscher, 2 Newfoundland, 2 great dane		computer database only		Stabej *et al*. (2005b)
			64	Irish Wolfhound	25 Irish Wolfhounds		Philipp *et al*. (2008a)
			38	Newfoundland	36 Newfoundlands		Wiersma *et al*. (2008)
SGCD	Y	N	25	Doberman Pinscher	10 Doberman Pinschers	Posafalvi *et al.* (2012), Andreasen *et al*. (2013)	Meurs *et al*. (2007)
			64	Irish Wolfhound	25 Irish Wolfhounds		Philipp *et al*. (2008a)
			38	Newfoundland	36 Newfoundlands		Wiersma *et al*. (2008)
			25	Doberman Pinscher	13 Doberman Pinschers		Stabej *et al.* (2005a)
STRN	N	Y	33	Boxer	16 Boxers	na	Meurs *et al.* (2013)
TAZ	Y	N	64	Irish Wolfhound	25 Irish Wolfhounds	Posafalvi *et al*. (2012)	Philipp *et al.* (2008a)
TCAP	Y	N	8	Irish Wolfhound	5 Irish Wolfhounds	Posafalvi *et al*. (2012), Andreasen *et al.* (2013)	Philipp *et al.* (2008b)
			38	Newfoundland	36 Newfoundlands		Wiersma *et al.* (2008)
			5	Doberman Pinscher	5 unspecified dogs without overt heart disease		O'Sullivan *et al.* (2011)
TMOD	N	N	64	Irish Wolfhound	25 Irish Wolfhounds	na	Philipp *et al*. (2008a)
TNNC1	Y	N	5	Doberman Pinscher	2 Labradors	Posafalvi *et al.* (2012), Andreasen *et al.* (2013)	Meurs *et al.* (2008)
TNNI3	Y	N	38	Newfoundland	36 Newfoundlands	Posafalvi *et al.* (2012), Andreasen *et al.* (2013)	Wiersma *et al.* (2008)
TNNT2	Y	N	5	Doberman Pinscher	2 Labradors	Posafalvi *et al.* (2012), Andreasen *et al.* (2013)	Meurs *et al.* (2008)
			5	Doberman Pinscher	5 unspecified dogs without overt heart disease		O'Sullivan *et al*. (2011)
			38	Newfoundland	36 Newfoundlands		Wiersma *et al.* (2008)
TPM1	Y	N	38	Newfoundland	36 Newfoundlands	Posafalvi *et al*. (2012)	Wiersma *et al*. (2008)
			5	Doberman Pinscher	5 unspecified dogs without overt heart disease		O'Sullivan *et al*. (2011)
TTN	Y	N	38	Newfoundland	36 Newfoundlands	Posafalvi *et al.* (2012), Andreasen *et al.* (2013)	Wiersma *et al.* (2008)
			2	Doberman Pinscher	5 mixed breeds		Oyama and Chittur (2005)
VCL	Y	N	5	Doberman Pinscher	5 unspecified dogs without overt heart disease	Posafalvi *et al.* (2012), Andreasen *et al*. (2013)	O'Sullivan *et al*. (2011)
			38	Newfoundland	36 Newfoundlands		Wiersma *et al*. (2008)

Y = yes, N = no

4. Genome Wide Association Studies (GWAS)

Genome wide association studies are a method of screening the genomes of many individuals for variants or regions that are associated with a trait (Manolio, 2010). Some variants will fall within genes and some outside of genes. When variants associated with a trait are found outside of genes it can be more difficult to establish their mode of action.

There have been three GWAS looking for an association with canine DCM. One of these led to the identification of a deletion in a splice site of *PDK4* associated with DCM in Doberman Pinschers (Meurs et al., 2012). A separate GWAS in Doberman Pinschers revealed a single SNP associated with DCM in a different location to the *PDK4* gene (Mausberg et al., 2011). The only other GWAS undertaken with regard to canine DCM is that by Philipp *et al.* (2012) which found one significantly associated SNP and five suggestively associated SNPs in Irish Wolfhounds. Of all the loci identified as associated with canine DCM only two are on the same chromosome, one of the Irish Wolfhound SNPs and the Striatin genes are both on chromosome 17, but even these are far apart. This indicates that there may be many loci involved in the development of canine DCM.

5. The Effects of Multiple Loci on DCM

Thus far in both canine and human genetic DCM studies loci have only been considered for an association with disease individually. There have been indications that multiple loci may influence the development of DCM (Mausberg et al., 2011). In human DCM where a panel of more than 50 loci are tested concurrently, often several loci are implicated. Simpson *et al.* (2015a) have shown theoretically that multiple loci affect the development of DCM in Doberman Pinschers. While this still requires validation, it is possible that similar effects occur in other breeds and species.

6. Power to Detect an Association with Canine DCM

The majority of studies undertaken with the aim of identifying causal genetic variants of canine DCM have only utilised small samples (5–40 individuals)

which is unlikely to be large enough to detect an effect. To establish appropriate study sizes and indicate the effect size that can be detected in published studies Power 3.1.7 Chi-squared goodness of fit tests were used (using the methods from Faul *et al.* (2009)). This takes known input parameters, including sample size, and calculates estimated effect sizes based on assumed power and can be used to indicate minimum sample size for prescribed power, alpha error rate, and effect size. This was done to indicate minimum sample sizes needed to detect various effect sizes (Figure 1).

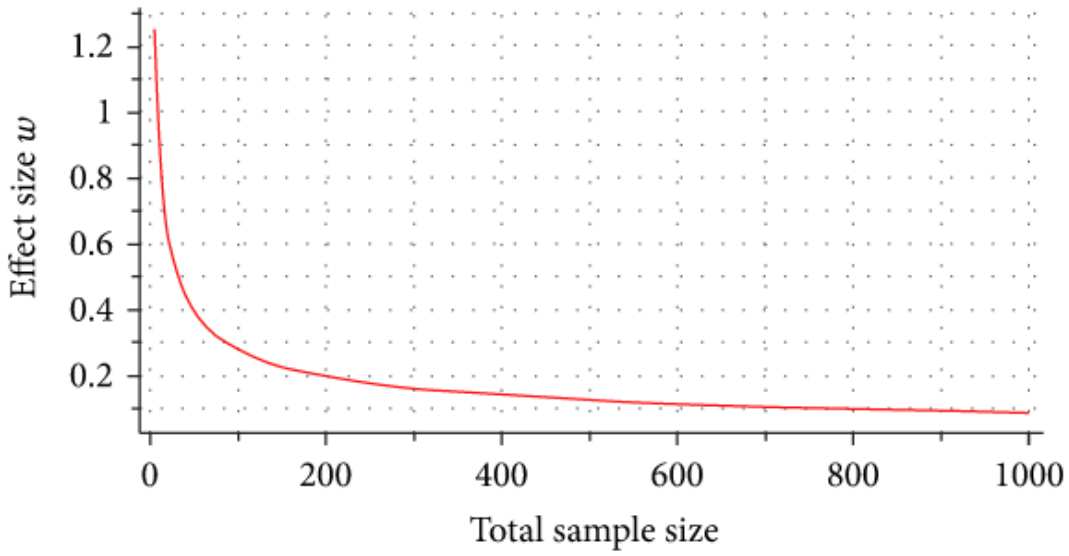


Figure 1. Goodness of fit tests: contingency tables Df = 1, err prob = 0.05, and power (err prob) = 0.8.

Published studies that have identified genetic variants associated with DCM have used sample sizes of 180 (Mausberg et al., 2011), 132 (Meurs et al., 2012), and 49 (Meurs et al., 2013). Assuming these studies had enough power to identify a positive effect (0.8), the effect sizes of these variants in these studies are 0.2088, 0.2438, and 0.4002, respectively, calculated using the sensitivity power analysis in Power 3.1.7 (Faul et al., 2009). These effect sizes, while not large, are larger than the standard effect size for small effect of 0.1. None of these variants explain all incidences of DCM, suggesting that other factors, which may be additional genetic variants of smaller effect, are involved. The sample size required to obtain a positive result from variants with small effect size (0.1) is 785, a number possibly not obtainable for all breeds but could

be aimed for in future studies. It is likely that earlier studies concentrated on simple Mendelian recessive, dominant traits and even a multiplicative risk models where Karlsson and Lindblad-Toh (2008) had suggested that affected and control groups of 20, 50, and 100, respectively, may suffice. Despite these suggestions, the authors indicated that higher group sizes (around 500 samples) would likely provide sufficient power to map an allele conferring a two-fold risk.

6.1. Discussion of Selected Breeds

While there are many breeds affected by canine DCM only a few have had genetic loci identified as associated with the disease. Here we discuss breeds with adolescent and adult onset DCM associated loci. The juvenile DCM that Portuguese water dogs develop is not discussed because it is already considered to be a distinct condition (Sleeper et al., 2002).

6.2. Boxers: Striatin (STRN)

The Boxer breed of dog was developed in the late 1800's primarily from the now extinct hunting dog the Bullenbeisser (Wagner, 1939). As with the development of most modern breeds there is documented evidence of inbreeding to produce the desired characteristics. In the case of the boxer this included a mating of a son to his mother, and following the creation of a breed standard in 1902 it is likely that usually Boxers will have exclusively been mated to other Boxers (Wagner, 1939). This limited genetic diversity is likely to have led to Boxers being prone to developing a number of diseases including heart disease, of which they frequently develop both arrhythmogenic right ventricular cardiomyopathy (ARVC) and DCM (Meurs et al., 2013). Since boxer cardiomyopathy was described by Harpster (1983) there have been several subtypes described, of the two displaying overt clinical symptoms these most closely align to human ARVC and DCM (Meurs et al., 2013). Recently Meurs *et al.* (2013) tested a deletion in the striatin (*STRN*) gene for an association with DCM in boxers. This deletion has previously been associated with ARVC and it was hypothesised that ARVC and DCM are variants of the same disease in Boxers and the homozygous genotype leads to DCM rather than ARVC (Meurs et al., 2013). They found a significant association with the deletion in its

homozygous form and DCM, but there were three cases of DCM where there was no deletion in the gene, thus indicating that there is at least one more cause of DCM in the breed to be established (Meurs et al., 2013).

6.3. Doberman Pinschers: *PDK4* and Chromosome 5 SNP

The Doberman Pinscher breed was developed at the end of the 1800's in Germany (Gruenig, 1939) when a number of individuals from established breeds were used to improve various characteristics. According to Gruenig (1939) these include the Manchester terrier, Greyhound, Rottweiler, Gordon Setter, Old English Sheepdog, Beauceron, Pinscher (probably German Pinscher), Weimaraner, and other less specific breeds such as Mastiff (possibly Great Dane), Hound, and Sporting dogs. The development of the breed happened rapidly, over a period of about 30 years, and since then Doberman Pinschers have only been bred to Doberman Pinschers (Gruenig, 1939), leading to a closed gene pool. Although a number of breeds contributed to the Doberman Pinscher it is likely that relatively few individuals of each breed were used likely leading to low genetic diversity. In addition to relatively few founders there is evidence of some individuals contributing a greater number of offspring to the breeding population than others (Gruenig, 1939).

Doberman Pinschers can develop a particularly severe type of DCM with rapid disease progression following the diagnosis of DCM with mean survival time of less than 10 weeks (Calvert et al., 1997, Petric et al., 2002). Poor survival time following diagnosis combined with the high prevalence of the disease with estimates ranging from 45% to 63% means DCM in this breed is a particular problem for clinicians (Wess et al., 2010). Doberman Pinschers display the fatty infiltration type of histopathology (Tidholm and Jonsson, 2005). Despite these poor statistics, age of onset of clinical signs is often later than in other commonly affected breeds (7.3 years in males and 8.6 years in females, compared to 4.8 (SD ± 2.3) years in Great Danes), giving individuals a good quality of life up until overt DCM clinical signs (Calvert et al., 1997, Meurs et al., 2001a). Across age groups there is no difference in clinical signs associated with DCM between the sexes including echocardiographic changes, presence and number of ventricular premature contractions, and overt DCM (Wess et al., 2010).

Unfortunately, however, males are more likely to have overt DCM than females with 73.7% of all observed males becoming clinically overt while only 26.3% of females observed became clinically overt (Wess et al., 2010).

DCM in Doberman Pinschers appears to be inherited in an autosomal dominant fashion with equal numbers of males and females affected, male-male transmission, and the mating of two affected individuals producing unaffected offspring (Meurs et al., 2007). There have been two loci identified as associated with DCM in the breed, a deletion of a splice site in pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*), and a SNP on chromosome 5 (Mausberg et al., 2011, Meurs et al., 2012). Unfortunately neither of these loci explains all incidences of DCM, and the *PDK4* deletion is not significantly associated with DCM in a separate Doberman Pinscher population (Owczarek-Lipska et al., 2013). There are still additional causes of DCM to be identified in Doberman Pinschers and the function of the SNP on chromosome 5 needs to be established.

6.4. German Short-Haired Pointers: Dystrophin (DMD)

The only gene associated with canine DCM in German short-haired pointers is Dystrophin (*DMD*) (Schatzberg et al., 1999). German short-haired pointers are not considered a breed particularly afflicted by heart disease and the deletion was only identified in two male litter mates (Egenvall et al., 2006, Schatzberg et al., 1999). This could be an isolated case which is unlikely to have implications in other breeds, particularly as the affected individuals also had skeletal myopathies, whereas in most cases of canine DCM there are not any other myopathies present (Schatzberg et al., 1999).

6.5. Irish Wolfhounds

Although Irish Wolfhounds have a long history, this includes a period when they were close to extinction. As part of conserving the breed, Great Danes, Scottish deerhounds, Borzoi, and Mastiffs were crossed with the few remaining Irish Wolfhounds (Samaha, 1991, Philipp et al., 2012). While this will have introduced some degree of genetic diversity to the breed, by necessity a large

amount of inbreeding will have been required to retain the Irish Wolfhound phenotype and so, like most modern breeds, genetic diversity is low (Jansson and Laikre, 2014).

Irish Wolfhounds do not usually develop a particularly severe form of DCM and with appropriate management can live with the disease for many months or years (Vollmar, 2000). Unfortunately, however, the prevalence of heart disease, including DCM, within the breed is very high, with 41% of individuals presenting with cardiac abnormalities, of which 58% have DCM (Vollmar, 2000). This high prevalence combined with early onset of clinical signs at around 4 years old (Distl et al., 2007) means that DCM in Irish Wolfhounds is of concern and so identifying genetic causes of the disease could have a large impact on the health of breed.

The mode of inheritance of DCM in Irish Wolfhounds has been shown to be autosomal dominant major gene effect, but with reduced penetrance indicating that multiple factors influence disease progression (Distl et al., 2007). Of the six SNPs associated with DCM in Irish Wolfhounds to date, only three lie within known genes (Philipp et al., 2012). Further work is therefore required to establish the functional significance of the alleles and to confirm the associations with DCM.

7. Conclusions: Impact of Genetics on Canine DCM

In the short term, the identification of the genetic contributors to DCM will enable targeted heart monitoring prior to the onset of clinical signs and clinical management of those dogs with increased risk of developing DCM. In the longer term, knowledge of the genetic factors which predispose to DCM will allow for selective breeding strategies to be considered and may identify novel therapeutic and diagnostic approaches. Individuals likely to develop DCM, identified through robust genetics, could be removed from breeding programmes with the ultimate goal of reducing the number of affected animals within the population and promoting the long term welfare of the breed. Understanding the genetic causes may also aid the stratification of distinct

clinical subtypes of DCM. This knowledge may also permit the development of novel DCM management programmes, help to guide prognosis, and assist with future drug and intervention research. Furthermore, investigations into causative genes in canine DCM may prove beneficial for other species, including humans. Novel mutations in canine breeds may serve as candidate genes in affected humans. For these reasons a more detailed understanding of the genetic basis of DCM in diverse dog breeds is now required.

Appendix 2 – Owner consent form for buccal swabs



The University of Nottingham

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SCHOOL OF VETERINARY MEDICINE AND SCIENCES

CANINE MOUTH/BUCCAL SAMPLE COLLECTION OWNER CONSENT FORM

The University of Nottingham is undertaking studies to improve the diagnosis and treatment of disease in dogs.

INFORMED CONSENT

I agree for a mouth (buccal) swab to be taken from my dog to be used in approved research conducted at the University of Nottingham, School of Veterinary Medicine and Science. The samples will be used in studies looking into the genetic causes of disease in dogs.

I understand that my dog's treatment (if any) will not be affected by being included in the study.

The current studies have been explained to me.

I understand that mouth swabs submitted to the University of Nottingham for research become entirely their property. I understand that the samples may also be used in future studies. I understand that the specific results from my pet may not be made available to me, but where possible general results will be made available as published papers and more concise understandable summaries.

I understand that this consent form covers all dogs that I own/have owned.

I understand that I can withdraw from the study by informing NottinghamCanineHealthGenomics@gmail.com and this will not affect my pet's treatment.

Owner name:_____

Signature:_____ **Date:**_____

Appendix 3 – Sample information form

A version of this form is available online to enable owners to keep us up to date on the health of their dogs. The link to this online version is: http://goo.gl/forms/8yEBQMRTMT



Canine genetics sample information form – University of Nottingham

Owner name:

Email:

Phone:

Breed:

Pet name:

Registered name:

Sex: MALE/FEMALE

Neutered: YES/NO

Age at neutering:

DOB/Age:

Father:

Mother:

Any current or previous medical/health problems:

Age/date of diagnosis:

Any drugs that the dog is on **now**, how long for and dose:

Any drugs that the dog **was** on, how long for and dose:

Sample Number (to be completed by Nottingham Genetics Team):

Appendix 4 – Questionnaire

Questionnaire to evaluate the risk factors that may lead to the development of Osteosarcoma, Cruciate ligament injury and heart disease in Rottweilers, Deerhounds and Wolfhounds (2015):

Section 1 - Owner and individual dog Information:

Name: _____

Address: _____

_____

Telephone Number: _____

Email Address: _____

N° of dogs of specific breed (IWH/Deerhound/Rottweiler) owned (not including puppies sold on):

_____

Name and breed of the Dog for which this questionnaire is being completed:

_____

KC registered (Please circle): **Yes** **No**

If Yes what is the registered KC name: _____

Dogs Date of birth or age: _____ **Years:** _____ **Months:** _____

Sex: _____ **Is this dog neutered (please circle):** **Yes** **No**

If yes when (at what age or date) was this dog neutered _____

Has your dog been used for breeding? (please circle) **Yes** **No**

What is your dog's current weight in kg (if possible could you check vet records or get them weighed?)

_____

Is your dog considered to be overweight either by you or your vet? (please circle) **Yes** **No**

If yes, please specify by how much (Please circle): Mild Moderate Substantial

Which of the following best describe your dog's temperament (please tick – can be more than one answer):

Aggressive and not approachable by other dogs and people they don't know well ☐

Can be unpredictable but generally fine with other dogs and people ☐

Very friendly and mixes very well with dogs and people ☐

Relaxed ☐

Highly strung ☐

Section 2 – Specific information about Osteosarcoma, cruciate ligament injury (CLI) and heart failure:

Has your dog ever suffered with osteosarcoma, CLI or heart failure?

Yes **No**

If your dog has osteosarcoma, CLI or heart failure please complete the section below – we appreciate this will be very emotional and thank you again for helping with this. If not then please proceed to section 3 to provide further information about your dog and their general health.

Has any relative of your dog suffered from osteosarcoma, CLI or heart failure – if yes state the relation:

_____

_____

_____

Section 2a - Osteosarcoma section:

What area of the body was affected by the osteosarcoma (please circle):

Left forelimb Right forelimb Left hind limb Right Hind limb Other:_____

What bone in that area was affected (if known)? (please circle):

Femur Tibia Fibula Humerus Radius Ulna Other:_____

What treatment plan did you go with?

Palliative care at home including pain relief and anti-inflammatories until euthanasia: ☐

Amputated the site of the bone cancer and then chemotherapy: ☐

Amputation alone: ☐

Chemotherapy alone: ☐

Radiotherapy alone: ☐

Please specify particular treatments:

_____

_____

_____

_____

What factors were involved in you making this treatment plan decision (e.g. life expectancy/prognosis, cost, home management, the invasive nature of the surgery, your dog's age?

Is / was your dog stable with this treatment regime? Yes No

If yes how long for? _____

Section 2b - Cruciate ligament injury:

Has you dog been diagnosed with CLI or any other orthopaedic problem? Yes No

If yes, when was this (detail for each separate problem):

Briefly provide details of the conditions (particular focus on CLI and whether this was partial or complete ligament tear):

How did the CLI occur (e.g. was the leg caught or trapped or was this an event with no obvious trauma?)

Has your dog had CLI in both hindlegs: Yes No

If yes, was the inciting cause the same for both legs (please detail)?

Did you dog have treatment (including surgery and hydrotherapy) for the CLI? Yes No

If yes please specify:

What factors influenced your decision over which treatment your dog received?

Specify any medication your dog is currently receiving for this (including nutritional supplements for this condition)

Section 2c - Heart disease questions:

Has your dog been diagnosed with any heart problems, such as a heart murmur or an abnormal heart rhythm?

Yes No

When was this identified?

Briefly give details:

Has your dog been diagnosed with heart failure? Yes No

When was this diagnosed?

Briefly give details:

_____

_____

_____

How long has your dog been receiving treatment for it's heart failure?

_____

What heart medication is your dog receiving?

- **Pimobendan (Vetmedin, Cardisure)** ☐
- **Furosemide (Dimazon, Frusecare, Frusedale, Frusol, Libeo)** ☐
- **ACE inhibitor (benazepril/Fortekor, Cardalis, Benefortin, Nelio, Prilben, Vetpril, enalapril/Enacard,Ramipril/Vasotop, imidapril/Prlium)** ☐
- **Sironolactone (Prilactone, Cardalis)** ☐

- **Other (please state)**

_____

Section 3 – Lifestyle and management section:

How many times per week does your dog take exercise?

Daily Every other day other (please specify) _____

If daily, how much exercise does your dog take each day?

Less than one hour 1-2 Hours 3-4 Hours More than 4 hours

If daily, how many times on average does your dog have structured exercise each day?

Once Twice Three Times Four or more

What type of exercise does your dog receive: for example gentle exercise on lead in flat open areas, or intense exercise such as working trials, agility or tracking or walks on hills / swimming?

Gentle Moderate Intense

Space for further details if required:

_____

_____

What is the predominant type of food you feed your dog and brand? (please circle and specify)

Dry complete food Tinned food Raw diet Cooked diet Other: _____

_____

_____

If you feed raw please state what raw meat you feed your dog and what is this balanced with:

If you feed raw meat what is your normal source of the meat?

Do you store this meat before feeding and if so for how long and is this refrigerated or frozen?

Do you routinely give your dog any dietary supplements such as those containing vitamins and minerals, joint supplements or fish oils?

Yes No

If yes please specify which:

Does your dog drink tap or bottled water? (please circle) Yes No

Does your dog drink other sources of water such as puddles, out of the toilet etc? Yes No

If yes please specify:

Do you treat your dog against fleas? Yes No

If 'no' please explain your reasons for this:

If 'yes' what is the name of the flea treatment that you use ?

How often do you treat your dog for fleas?

Do you treat your dog against worms? Yes No

If 'yes' which worming products do you give your dog?

How often do you worm your dog?

Section 4 – Health section:

Does your dog suffer with any chronic (on going/longstanding) problems such as diarrhoea, vomiting or coughing?

Yes No

If yes please specify which, along with the approximate frequency and whether they are receiving any treatment for this (intermittently or continuously):

Has your dog ever suffered any trauma from for example a road traffic accident, the result of a fight with another dog, or any other accident that caused physical injury?

Yes No

If yes please briefly specify what happened:

Section 5 – Veterinary information:

Is your dog vaccinated? Yes No

Is the vaccine course up to date? Yes No

Has your dog received a course of anti-inflammatory or pain relief medication in the past for more than a week?

Yes No

If yes please specify which:

Has your dog received courses of antibiotics for more than a week? Yes No

If yes please specify:

Has your dog ever broken or injured a bone or injured a joint?

Yes No

If yes where was the break or injury and specify details?

Has your dog had any surgery on a bone or a joint (not including as part of treatment for cruciate ligament injury)

Yes No

If yes please specify:

_____

_____

_____

If your dog had surgery did it have any implants, plates or prosthetics as part of the repair technique?

Yes No

If yes please specify:

_____

_____

_____

Has your dog ever had an X-ray, a CT scan, an MRI or radiotherapy? Yes No

If yes please specify which and what this was for:

_____

_____

_____

Has your dog ever received chemotherapy (for a particular cancer or immune-mediated disease)?

Yes No

If so please give brief details of why and what they received:

_____

_____

_____

Date Questionnaire completed: _____

Thank you for taking the time to complete this questionnaire. Your input into the Nottingham breed health project is invaluable and greatly appreciated.

Appendix 5 – Supplementary tables from "A predictive model for canine dilated cardiomyopathy – a meta-analysis of Doberman Pinscher data", Chapter 3

Supplementary Table 1. The phenotype decisions, combined genotype frequencies and predicted number of individuals for each genotype combination from the model incorporating the two known DCM loci.

PDK4		Chr5 SNP		Combined genotype freq	Predicted number of individuals	Phenotype
genotype	freq	genotype	freq			
Wt Wt	0.72	TT	0.74	0.5328	96.9696	Healthy
Wt Wt	0.72	TC	0.24	0.1728	31.4496	Healthy
Wt Wt	0.72	CC	0.02	0.0144	2.6208	DCM
Wt del	0.26	TT	0.74	0.1924	35.0168	Healthy
Wt del	0.26	TC	0.24	0.0624	11.3568	DCM
Wt del	0.26	CC	0.02	0.0052	0.9464	DCM
Del del	0.02	TT	0.74	0.0148	2.6936	Healthy
Del del	0.02	TC	0.24	0.0048	0.8736	DCM
Del del	0.02	CC	0.02	0.0004	0.0728	DCM

Supplementary Table 2. The phenotype decisions, combined genotype frequencies and predicted number of individuals for each genotype combination incorporating the two known DCM loci + 50% (males) more susceptible to DCM.

sex		*PDK4*		Chr5 SNP		combined genotype freq	Predicted number of individuals	Phenotype
sex	freq	genotype	freq	genotype	freq			
female	0.5	Wt Wt	0.72	TT	0.74	0.2664	48.4848	Healthy
female	0.5	Wt Wt	0.72	TC	0.24	0.0864	15.7248	Healthy
female	0.5	Wt Wt	0.72	CC	0.02	0.0072	1.3104	DCM
female	0.5	Wt del	0.26	TT	0.74	0.0962	17.5084	Healthy
female	0.5	Wt del	0.26	TC	0.24	0.0312	5.6784	DCM
female	0.5	Wt del	0.26	CC	0.02	0.0026	0.4732	DCM
female	0.5	Del del	0.02	TT	0.74	0.0074	1.3468	Healthy
female	0.5	Del del	0.02	TC	0.24	0.0024	0.4368	DCM
female	0.5	Del del	0.02	CC	0.02	0.0002	0.0364	DCM
male	0.5	Wt Wt	0.72	TT	0.74	0.2664	48.4848	Healthy
male	0.5	Wt Wt	0.72	TC	0.24	0.0864	15.7248	DCM
male	0.5	Wt Wt	0.72	CC	0.02	0.0072	1.3104	DCM
male	0.5	Wt del	0.26	TT	0.74	0.0962	17.5084	DCM
male	0.5	Wt del	0.26	TC	0.24	0.0312	5.6784	DCM
male	0.5	Wt del	0.26	CC	0.02	0.0026	0.4732	DCM
male	0.5	Del del	0.02	TT	0.74	0.0074	1.3468	DCM
male	0.5	Del del	0.02	TC	0.24	0.0024	0.4368	DCM
male	0.5	Del del	0.02	CC	0.02	0.0002	0.0364	DCM

Supplementary Table 3. The phenotype decisions, combined genotype frequencies and predicted number of individuals for each genotype combination from the model incorporating the two known DCM loci + an additional autosomal dominant DCM susceptibility locus.

additional autosomal dominant locus		*PDK4*		Chr5 SNP		combined genotype freq	Predicted number of individuals	Phenotype
genotype	freq	genotype	freq	genotype	freq			
good good	0.490	Wt Wt	0.72	TT	0.74	0.261072	47.515104	Healthy
good good	0.490	Wt Wt	0.72	TC	0.24	0.084672	15.410304	Healthy
good good	0.490	Wt Wt	0.72	CC	0.02	0.007056	1.284192	DCM
good good	0.490	Wt del	0.26	TT	0.74	0.094276	17.158232	Healthy
good good	0.490	Wt del	0.26	TC	0.24	0.030576	5.564832	DCM
good good	0.490	Wt del	0.26	CC	0.02	0.002548	0.463736	DCM
good good	0.490	Del del	0.02	TT	0.74	0.007252	1.319864	Healthy
good good	0.490	Del del	0.02	TC	0.24	0.002352	0.428064	DCM
good good	0.490	Del del	0.02	CC	0.02	0.000196	0.035672	DCM
good bad	0.420	Wt Wt	0.72	TT	0.74	0.223776	40.727232	DCM
good bad	0.420	Wt Wt	0.72	TC	0.24	0.072576	13.208832	DCM
good bad	0.420	Wt Wt	0.72	CC	0.02	0.006048	1.100736	DCM
good bad	0.420	Wt del	0.26	TT	0.74	0.080808	14.707056	DCM
good bad	0.420	Wt del	0.26	TC	0.24	0.026208	4.769856	DCM
good bad	0.420	Wt del	0.26	CC	0.02	0.002184	0.397488	DCM
good bad	0.420	Del del	0.02	TT	0.74	0.006216	1.131312	DCM
good bad	0.420	Del del	0.02	TC	0.24	0.002016	0.366912	DCM
good bad	0.420	Del del	0.02	CC	0.02	0.000168	0.030576	DCM
bad bad	0.090	Wt Wt	0.72	TT	0.74	0.047952	8.727264	DCM
bad bad	0.090	Wt Wt	0.72	TC	0.24	0.015552	2.830464	DCM
bad bad	0.090	Wt Wt	0.72	CC	0.02	0.001296	0.235872	DCM
bad bad	0.090	Wt del	0.26	TT	0.74	0.017316	3.151512	DCM
bad bad	0.090	Wt del	0.26	TC	0.24	0.005616	1.022112	DCM
bad bad	0.090	Wt del	0.26	CC	0.02	0.000468	0.085176	DCM
bad bad	0.090	Del del	0.02	TT	0.74	0.001332	0.242424	DCM
bad bad	0.090	Del del	0.02	TC	0.24	0.000432	0.078624	DCM
bad bad	0.090	Del del	0.02	CC	0.02	0.000036	0.006552	DCM

Supplementary Table 4. Phenotype decisions, genotype frequencies and predicted number of individuals for each genotype combination incorporating the two known DCM loci + an autosomal additive DCM susceptibility locus.

additional autosomal additive locus		*PDK4*		Chr5 SNP		combined genotype freq	Predicted number of individuals	Phenotype
genotype	freq	genotype	freq	genotype	freq			
good good	0.490	Wt Wt	0.72	TT	0.74	0.261072	47.515104	Healthy
good good	0.490	Wt Wt	0.72	TC	0.24	0.084672	15.410304	Healthy
good good	0.490	Wt Wt	0.72	CC	0.02	0.007056	1.284192	DCM
good good	0.490	Wt del	0.26	TT	0.74	0.094276	17.158232	Healthy
good good	0.490	Wt del	0.26	TC	0.24	0.030576	5.564832	DCM
good good	0.490	Wt del	0.26	CC	0.02	0.002548	0.463736	DCM
good good	0.490	Del del	0.02	TT	0.74	0.007252	1.319864	Healthy
good good	0.490	Del del	0.02	TC	0.24	0.002352	0.428064	DCM
good good	0.490	Del del	0.02	CC	0.02	0.000196	0.035672	DCM
good bad	0.420	Wt Wt	0.72	TT	0.74	0.223776	40.727232	Healthy
good bad	0.420	Wt Wt	0.72	TC	0.24	0.072576	13.208832	DCM
good bad	0.420	Wt Wt	0.72	CC	0.02	0.006048	1.100736	DCM
good bad	0.420	Wt del	0.26	TT	0.74	0.080808	14.707056	DCM
good bad	0.420	Wt del	0.26	TC	0.24	0.026208	4.769856	DCM
good bad	0.420	Wt del	0.26	CC	0.02	0.002184	0.397488	DCM
good bad	0.420	Del del	0.02	TT	0.74	0.006216	1.131312	DCM
good bad	0.420	Del del	0.02	TC	0.24	0.002016	0.366912	DCM
good bad	0.420	Del del	0.02	CC	0.02	0.000168	0.030576	DCM
bad bad	0.090	Wt Wt	0.72	TT	0.74	0.047952	8.727264	DCM
bad bad	0.090	Wt Wt	0.72	TC	0.24	0.015552	2.830464	DCM
bad bad	0.090	Wt Wt	0.72	CC	0.02	0.001296	0.235872	DCM
bad bad	0.090	Wt del	0.26	TT	0.74	0.017316	3.151512	DCM
bad bad	0.090	Wt del	0.26	TC	0.24	0.005616	1.022112	DCM
bad bad	0.090	Wt del	0.26	CC	0.02	0.000468	0.085176	DCM
bad bad	0.090	Del del	0.02	TT	0.74	0.001332	0.242424	DCM
bad bad	0.090	Del del	0.02	TC	0.24	0.000432	0.078624	DCM
bad bad	0.090	Del del	0.02	CC	0.02	0.000036	0.006552	DCM

Supplementary Table 5. The phenotype decisions, combined genotype frequencies and predicted number of individuals for each genotype combination from the model incorporating the two known DCM loci + an additional autosomal recessive DCM susceptibility locus.

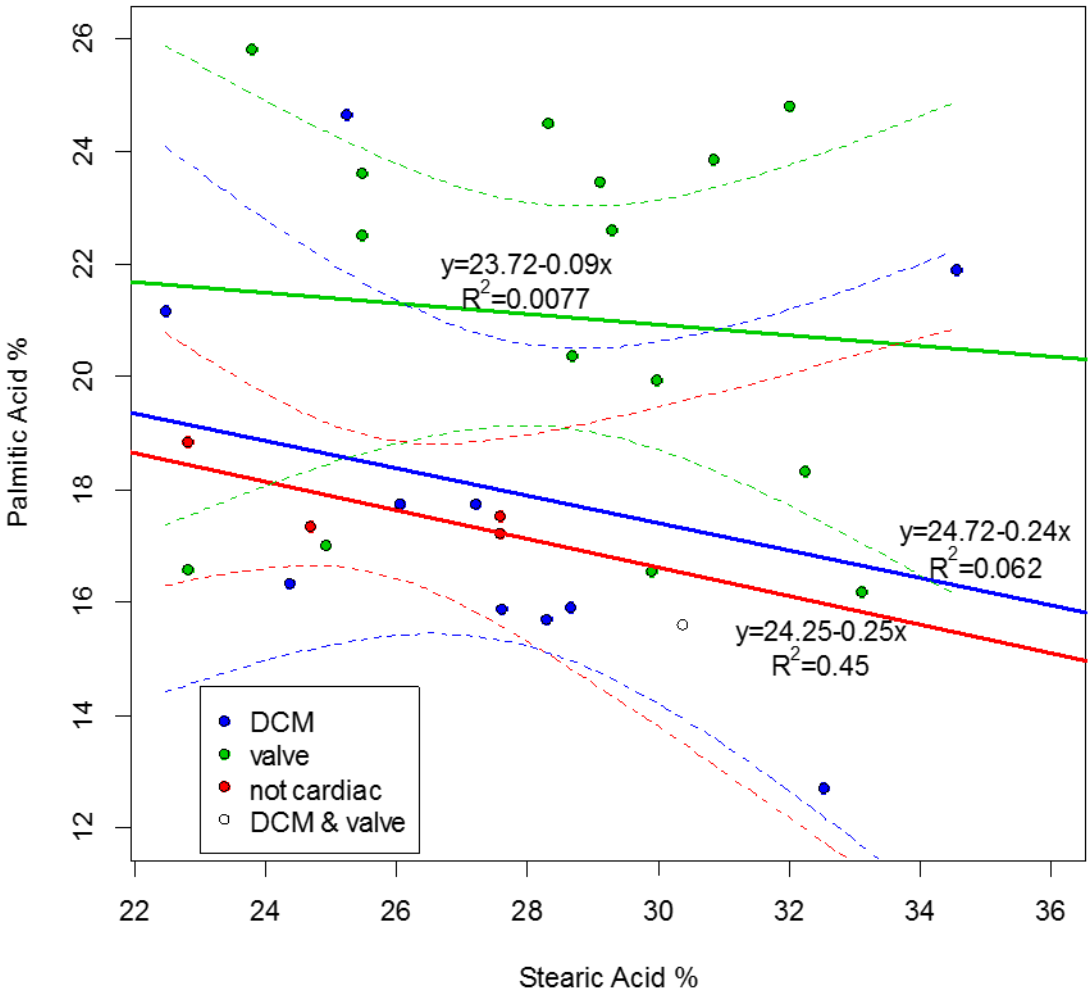
additional autosomal recessive locus		*PDK4*		Chr5 SNP		combined genotype freq	Predicted number of individuals	Phenotype
genotype	freq	genotype	freq	genotype	freq			
good good	0.490	Wt Wt	0.72	TT	0.74	0.261072	47.515104	Healthy
good good	0.490	Wt Wt	0.72	TC	0.24	0.084672	15.410304	Healthy
good good	0.490	Wt Wt	0.72	CC	0.02	0.007056	1.284192	DCM
good good	0.490	Wt del	0.26	TT	0.74	0.094276	17.158232	Healthy
good good	0.490	Wt del	0.26	TC	0.24	0.030576	5.564832	DCM
good good	0.490	Wt del	0.26	CC	0.02	0.002548	0.463736	DCM
good good	0.490	Del del	0.02	TT	0.74	0.007252	1.319864	Healthy
good good	0.490	Del del	0.02	TC	0.24	0.002352	0.428064	DCM
good good	0.490	Del del	0.02	CC	0.02	0.000196	0.035672	DCM
good bad	0.420	Wt Wt	0.72	TT	0.74	0.223776	40.727232	Healthy
good bad	0.420	Wt Wt	0.72	TC	0.24	0.072576	13.208832	Healthy
good bad	0.420	Wt Wt	0.72	CC	0.02	0.006048	1.100736	DCM
good bad	0.420	Wt del	0.26	TT	0.74	0.080808	14.707056	Healthy
good bad	0.420	Wt del	0.26	TC	0.24	0.026208	4.769856	DCM
good bad	0.420	Wt del	0.26	CC	0.02	0.002184	0.397488	DCM
good bad	0.420	Del del	0.02	TT	0.74	0.006216	1.131312	Healthy
good bad	0.420	Del del	0.02	TC	0.24	0.002016	0.366912	DCM
good bad	0.420	Del del	0.02	CC	0.02	0.000168	0.030576	DCM
bad bad	0.090	Wt Wt	0.72	TT	0.74	0.047952	8.727264	DCM
bad bad	0.090	Wt Wt	0.72	TC	0.24	0.015552	2.830464	DCM
bad bad	0.090	Wt Wt	0.72	CC	0.02	0.001296	0.235872	DCM
bad bad	0.090	Wt del	0.26	TT	0.74	0.017316	3.151512	DCM
bad bad	0.090	Wt del	0.26	TC	0.24	0.005616	1.022112	DCM
bad bad	0.090	Wt del	0.26	CC	0.02	0.000468	0.085176	DCM
bad bad	0.090	Del del	0.02	TT	0.74	0.001332	0.242424	DCM
bad bad	0.090	Del del	0.02	TC	0.24	0.000432	0.078624	DCM
bad bad	0.090	Del del	0.02	CC	0.02	0.000036	0.006552	DCM

Supplementary Table 6. The phenotype decisions, combined genotype frequencies and predicted number of individuals for each genotype combination from the model incorporating the two known DCM loci + an additional X-linked DCM susceptibility locus, where X is normal and x is susceptible.

additional X locus		*PDK4*		Chr5 SNP		combined genotype freq	Predicted number of individuals	Phenotype
genotype	freq	genotype	freq	genotype	freq			
XY	0.350	Wt Wt	0.72	TT	0.74	0.18648	33.93936	Healthy
XY	0.350	Wt Wt	0.72	TC	0.24	0.06048	11.00736	Healthy
XY	0.350	Wt Wt	0.72	CC	0.02	0.00504	0.91728	DCM
XY	0.350	Wt del	0.26	TT	0.74	0.06734	12.25588	Healthy
XY	0.350	Wt del	0.26	TC	0.24	0.02184	3.97488	DCM
XY	0.350	Wt del	0.26	CC	0.02	0.00182	0.33124	DCM
XY	0.350	Del del	0.02	TT	0.74	0.00518	0.94276	Healthy
XY	0.350	Del del	0.02	TC	0.24	0.00168	0.30576	DCM
XY	0.350	Del del	0.02	CC	0.02	0.00014	0.02548	DCM
xY	0.150	Wt Wt	0.72	TT	0.74	0.07992	14.54544	DCM
xY	0.150	Wt Wt	0.72	TC	0.24	0.02592	4.71744	DCM
xY	0.150	Wt Wt	0.72	CC	0.02	0.00216	0.39312	DCM
xY	0.150	Wt del	0.26	TT	0.74	0.02886	5.25252	DCM
xY	0.150	Wt del	0.26	TC	0.24	0.00936	1.70352	DCM
xY	0.150	Wt del	0.26	CC	0.02	0.00078	0.14196	DCM
xY	0.150	Del del	0.02	TT	0.74	0.00222	0.40404	DCM
xY	0.150	Del del	0.02	TC	0.24	0.00072	0.13104	DCM
xY	0.150	Del del	0.02	CC	0.02	0.00006	0.01092	DCM
Females (XX)		***PDK4***		**Chr5 SNP**				**Phenotype**
XX	0.25	Wt Wt	0.72	TT	0.74	0.1332	24.2424	Healthy
XX	0.25	Wt Wt	0.72	TC	0.24	0.0432	7.8624	Healthy
XX	0.25	Wt Wt	0.72	CC	0.02	0.0036	0.6552	DCM
XX	0.25	Wt del	0.26	TT	0.74	0.0481	8.7542	Healthy
XX	0.25	Wt del	0.26	TC	0.24	0.0156	2.8392	DCM
XX	0.25	Wt del	0.26	CC	0.02	0.0013	0.2366	DCM
XX	0.25	Del del	0.02	TT	0.74	0.0037	0.6734	Healthy
XX	0.25	Del del	0.02	TC	0.24	0.0012	0.2184	DCM
XX	0.25	Del del	0.02	CC	0.02	0.0001	0.0182	DCM
Xx	0.210	Wt Wt	0.72	TT	0.74	0.111888	20.363616	Healthy
Xx	0.210	Wt Wt	0.72	TC	0.24	0.036288	6.604416	DCM
Xx	0.210	Wt Wt	0.72	CC	0.02	0.003024	0.550368	DCM
Xx	0.210	Wt del	0.26	TT	0.74	0.040404	7.353528	DCM
Xx	0.210	Wt del	0.26	TC	0.24	0.013104	2.384928	DCM
Xx	0.210	Wt del	0.26	CC	0.02	0.001092	0.198744	DCM
Xx	0.210	Del del	0.02	TT	0.74	0.003108	0.565656	DCM
Xx	0.210	Del del	0.02	TC	0.24	0.001008	0.183456	DCM
Xx	0.210	Del del	0.02	CC	0.02	0.000084	0.015288	DCM

xx	0.050	Wt Wt	0.72	TT	0.74	0.02664	4.84848	DCM
xx	0.050	Wt Wt	0.72	TC	0.24	0.00864	1.57248	DCM
xx	0.050	Wt Wt	0.72	CC	0.02	0.00072	0.13104	DCM
xx	0.050	Wt del	0.26	TT	0.74	0.00962	1.75084	DCM
xx	0.050	Wt del	0.26	TC	0.24	0.00312	0.56784	DCM
xx	0.050	Wt del	0.26	CC	0.02	0.00026	0.04732	DCM
xx	0.050	Del del	0.02	TT	0.74	0.00074	0.13468	DCM
xx	0.050	Del del	0.02	TC	0.24	0.00024	0.04368	DCM
xx	0.050	Del del	0.02	CC	0.02	0.00002	0.00364	DCM

Appendix 6 – Plot of palmitic acid vs stearic acid with associated regression lines.



95% confidence intervals are shown as dashed lines. Individuals with a DCM diagnosis are shown in blue, individuals with a valve disease diagnosis are shown in green, and individuals with other diagnoses are shown in red. The non-coloured point indicates the individual which had its diagnosis recorded as "DCM/end stage valve disease".

Appendix 7 – PIPS Reflective Statement

Note to examiners:

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

PIPS Reflective Statement

My internship was at NHiS Ltd in Nottingham. During my internship I analysed Hospital Episode Statistics (HES) data to establish patterns with the aim of identifying areas where the care of patients with particular illnesses could be improved. My main focus was on co-morbidities in patients with multiple sclerosis. The work that I did on this contributed towards a report "Measuring the burden of hospitalisation in multiple sclerosis: A cross-sectional analysis of the English Hospital Episode Statistics database 2009-2014" which was completed and published once I had left my internship. In addition to interrogating the multiple sclerosis data I also analysed some spinal injury data and contributed towards two other reports by analysing small amounts of data and proof reading whilst I was on my internship.

I had not previously worked in such a commercially driven environment so the experience was valuable as it allowed me to understand the commercial sector better than I did before my internship. Something that was particularly new to me was a visit by the CEO of the PLC that owns the company. I had not previously met a CEO or been exposed to such corporate language.

I was the first DTP internship student that NHiS Ltd had taken on and due to the success of my internship they have since taken on at least two more DTP internship students. I am pleased that other DTP students are also getting a good internship experience with NHiS Ltd.

An unexpected outcome of my internship is that a formal collaboration has emerged between my PhD supervisors and NHiS Ltd. This came about because I recognised how useful HES data could be in supporting a wide range of medical research so I put both parties in contact with each other.

My internship helped me to recognise that I have a broader range of skills to offer than I had previously thought. In terms of my career my internship confirmed that I would like to stay within academia, but that there are opportunities outside of academia which could also suit my abilities and interests so I have not fully excluded a career outside of academia.